SCREENING FOR LYSOSOMAL STORAGE DISEASE STATUS

FIELD OF THE INVENTION

This invention relates to screening to ascertain the nature or status of lysosomal storage disorders (LSD) and in particular by the use of lipid containing storage associated compounds

BACKGROUND OF THE INVENTION

Most lysosomal storage disorders (LSD) are inherited in an autosomal recessive manner with the exception of Fabry disease, Danon disease and mucopolysaccharidosis (MPS) type II, which display X-linked recessive inheritance. Some LSD have been classified into clinical subtypes (such as the Hurler/Scheie variants of MPS I, or the infantile/juvenile/adult onset forms of Pompe disease), but it is clear that most LSD have a broad continuum of clinical severity and age of presentation. With the advent of molecular biology/genetics and the characterisation of many of the LSD genes, it is now recognised that the range of severity may, in part, be ascribed to different mutations within the same gene. However, genotype/phenotype correlations do not always hold and other factors including genetic background and environmental factors, presumably play a role in disease progression.

LSD are rare disorders with incidences ranging from about 1:50,000 births to less than 1:4,000,000 births (1). However, when considered as a group, the combined incidence is substantially higher. We have previously estimated the prevalence of LSD in Australia to be 1:7,700 births, excluding the neuronal ceroid lipofuscinoses. The prevalence of this latter group of LSD has been reported to be as high as 1 per 12,500 births in the United States (2). In Finland, incidence values of 1 per 13,000 births for infantile and 1 per 21,000 births for juvenile forms have been reported (3). Clearly, the neuronal ceroid lipofuscinoses will contribute significantly to the overall prevalence of LSD. It is equally certain that additional LSD will be identified as our understanding of lysosomal biology and the clinical manifestations resulting from lysosomal dysfunction improve. A conservative estimate of the prevalence of LSD in the Australian population would be 1 in 5,000 births.

Inborn errors of metabolism causing lysosomal storage have well-recognised effects on neuronal function. In many of the LSD almost all patients develop central nervous system (CNS) dysfunction while in a few disorders such as MPS IVA and MPS VI there are no reports of CNS involvement. In a number of other disorders, notably Gaucher disease, Niemann-Pick disease, MPS I and MPS II, the range of clinical severity spans individuals with no CNS involvement to those with severe CNS pathology. Notwithstanding the diverse clinical manifestations within LSD, the majority of patients will develop CNS disease.

One of the main determining factors of LSD severity is the residual activity of the affected enzyme. Kinetic models that describe correlations between residual enzyme activity and the turnover rate of its substrate have been proposed (4). Such a mathematical model has been tested in skin fibroblasts and residual activity of β-hexosaminidase A and arylsulphatase A correlated well with substrate turnover (5). However, for many LSD residual enzyme activity is difficult to measure accurately and even when such measurements can be performed they are not always reflective of disease severity, especially CNS pathology. We propose that the level of stored substrates in particular cells or tissues in these disorders, as well as perhaps the levels of secondary metabolites, will reflect disease severity and is likely to yield additional information about the pathophysiology in LSD. The key in determining the absence or presence of CNS pathology lies in understanding the pathogenic process of LSD, which at present is poorly understood.

Unless the context requires otherwise, the word "comprise," or variations such as "comprises" or "comprising" mean the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

SUMMARY OF THE INVENTION

It has been found that use of estimates of the relative levels of LSD (Lysosomal Storage Disorder) storage associated compounds in body tissues or fluids can be used to assess the LSD status of an individual.

In a first broad form of a first aspect the invention could be said to reside in a method of assessing an LSD status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of three or more compound indicators, said indicators being indicative of the level of respectively each of three or more lipid containing storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual.

In a first broad form of a second aspect the invention could be said to reside in a method of assessing an LSD status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of two or more compound indicators being indicative of the level respectively of each of two or more lipid containing storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual,

the two or more storage associated compounds selected to discriminate between an LSD individual from a non-LSD individual with an acceptable confidence level.

In a first broad form of a third aspect the invention could be said to reside in a method for screening for the status of two or more LSDs in an individual,

taking a single tissue or body fluid sample from the individual,

estimating a level in the sample of each three or more compound indicators being indicative of the concentration respectively of each of three or more lipid containing storage associated compounds,

calculating a first LSD index number using a first set of two or more of said compound indicators and comparing the first LSD index number of the sample with a first control indicator to provide an assessment of the LSD status of the first LSD,

and calculating a second LSD index number using a second set of two or more of said compound indicators and comparing the second LSD index number of the individual with a second standard to provide an assessment of the LSD status of the second LSD in the individual.

In a first broad form of a fourth form the invention might be said to reside in a method of developing a diagnostic method comprising the steps of

taking a first group of LSD samples one each from a plurality of LSD individuals affected by one type of LSD,

taking a second group of control samples one each from a plurality of control individuals not affected by LSD

the sample being of a tissue or body fluid of the control individuals and LSD group of individuals

interrogating the first group of samples by mass spectrometry for first levels of a plurality of indicators of respective storage associated compounds,

interrogating the second group of samples by mass spectrometry for second levels of the plurality of indicators of respective storage associated compounds,

the storage associated compounds selected from the class of compounds consisting of the group glycolipids and phospholipids,

comparing the first levels with the second levels

identifying a first group of storage associated compound which are shown as having increased levels of indicators in the first LSD group compared to the control group,

identifying a second group of storage associated compounds which are shows as having decreased levels of indicators in the LSD group compared to the control group,

formulating a combination of two or more of the first and/or second group of indicators by which to calculate and index number whereby to distinguish LSD samples from control samples, and preferably

preparing a standard being a scale of index numbers reflective of the severity of the LSD.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Glycolipid levels in Dried Blood Spots. Box plots showing the relative levels of glucosylceramide (panel A) and lactosylceramide (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 2. Glycolipid levels in Dried Blood Spot. Box plots showing the relative levels of ceramide (panel A) and sphingomyelin (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 3. Glycolipid Ratios in Dried Blood Spots. Box plots showing the ratios of glucosylceramide to lactosylceramide (panel A) and ceramide to sphingomyelin (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 4. Glycolipid Analysis in Dried Blood Spots. Box plots showing the ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) in dried blood spots from control (1), Gaucher patients on enzyme therapy (2) and Gaucher patients not on therapy (3). The centre bar shows the median value, the box denotes the 25th and 75th centiles and the upper and lower bars represent the range. Open circles and stars represent outliers and extreme outliers respectively. N= the number of samples in each group.

Figure 5. Relative lipid levels in dried blood spots from treated and untreated Gaucher disease patients. Relative glucosylceramide (panel A) and ceramide (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The shaded area shows the normal range for each analyte.

Figure 6. Relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients. The ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The shaded area shows the normal range for each ratio or function.

Figure 7. Correlation between relative lipid levels in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. Glucosylceramide (panel A) and ceramide (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The lipid

levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 8. Correlation between relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. Glucosylceramide: lactosylceramide ratio (panel A) and ceramide:sphingomyelin ratio (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The lipid levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 9. Correlation between relative lipid ratios in dried blood spots from treated and untreated Gaucher disease patients and chitotriosidase values. The ratio of (glucosylceramide x ceramide) / (lactosylceramide x sphingomyelin) (panel A) and a discriminate function of the same four analytes (panel B) were determined in dried blood spots from patients that were either untreated or had been receiving enzyme replacement therapy for up to 130 months. The lipid levels were related to the chitotriosidase levels determined in the same patients at the same time.

Figure 10. Lipid concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ by the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25th and 75th centiles (boxes) and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 11. Lactosylceramide and trihexosylceramide concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were

analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the LC and CTH species. Fabry Het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes. Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Figure 12. Lipid ratios in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected lipid type (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 13. Individual lipid species in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid species was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected lipid species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 14. Selected lipid species concentrations in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the lipid species. Fabry het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes.

Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Figure 15. Selected lipids and proteins in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the lipid ratios and saposin C. Fabry het (affected) patients were heterozygotes who had been diagnosed with clinical symptoms of Fabry disease; clinical details were not available for the other heterozygotes. Two of the Fabry patients were known to have undergone renal transplants (Fabry (RT)).

Ratio 4 = (LC C24:1*CTH C24:1)/(GC C24:0*SM C24:0) all species corrected for PC.

Figure 16. Individual PC species in urine from controls, Fabry and Fabry heterozygotes. Urine samples (1.5 mL) were extracted with $CHCl_3$ using the method of Bligh/Dyer. Lipids were analysed by tandem mass spectrometry as described previously. Each lipid species was corrected for the total PC concentration in that sample. The box plots show the median levels of each corrected lipid species (centre bar), the 25^{th} and 75^{th} centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 17. Lipid concentrations in plasma from controls, Fabry and Fabry heterozygotes. Plasma samples (100 μL) were extracted with $CHCl_3$ using the method of Folsch. Lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25^{th} and 75^{th} centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 18. Lipid species in plasma from controls, Fabry and Fabry heterozygotes. Plasma samples (100 μL) were extracted with $CHCl_3$ using the method of Folsch. Lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the different lipid species.

Figure 19. Lipid concentrations in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid type (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 20. Lipid species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each lipid species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 21. CTH species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The box plots show the median levels of each CTH species (centre bar), the 25th and 75th centiles (boxes), and the upper and lower limits (upper and lower bars). The circles and stars represent outliers and extreme outliers respectively.

Figure 22. Lipid species in whole blood from controls, Fabry and Fabry heterozygotes. Dried blood spots (2 x 3 mm) were extracted with isopropanol and the lipids were analysed by tandem mass spectrometry as described previously. The scatter plots show the relationship between the different lipid species.

Figure 23. Plasma CTH levels in controls, Fabry hemizygotes, Fabry hemizygotes on ERT, Fabry heterozygotes and Fabry heterozygotes on ERT. The bar represents the median value, the box represents the 25th to 75th centiles and the upper and lower bars represent the range. Circles and stars represent outliers and extreme outliers, respectively. N = sample numbers in each group.

Figure 24. Plasma lipid levels in controls, Fabry hemizygotes, Fabry hemizygotes on ERT, Fabry heterozygotes and Fabry heterozygotes on ERT. The bar represents the median value, the box represents the 25th to 75th centiles and the upper and lower bars represent the range. Circles and stars represent outliers and extreme outliers, respectively. N = sample numbers in each group.

Figure 25. Plasma lipid levels in controls, Fabry hemizygotes, Fabry hemizygotes on ERT, Fabry heterozygotes and Fabry heterozygotes on ERT.

Figure 26. Urine lipid levels in controls, Fabry hemizygotes, Fabry hemizygotes on ERT, Fabry heterozygotes and Fabry heterozygotes on ERT. The bar represents the median value, the box represents the 25th to 75th centiles and the upper and lower bars represent the range. Circles and stars represent outliers and extreme outliers, respectively. N = sample numbers in each group.

Figure 27. Urine lipid ratios in controls, Fabry hemizygotes, Fabry hemizygotes on ERT, Fabry heterozygotes and Fabry heterozygotes on ERT.

DETAILED DESCRIPTION OF THE ILLUSTRATED AND EXEMPLIED EMBODIMENTS OF THE INVENTION

Lysosomes are organelles in eukaryotic cells that function in the degradation of macromolecules, including glycosphingolipids, glycogen, mucopolysaccharides, oligosaccharides, aminoglycans, phospholipids and glycoproteins, into component parts that can be reused in biosynthetic pathways or discharged by cells as waste. The metabolism of exo- and endogenous high molecular weight compounds normally occurs in the lysosomes, and the process is normally regulated in a stepwise process by degradation enzymes. However, when a lysosomal enzyme is not present in the lysosome or does not function properly, the enzymes specific macromolecular substrate accumulates in the lyosome as "storage material" causing a variety of diseases, collectively known as lysosomal storage diseases. In each of these diseases, lysosomes are unable to degrade a specific compound or group of compounds because the enzyme that catalyzes a specific degradation reaction is missing from the lysosome or is present in low concentrations or has been altered.

The field of lysosomal storage disorders is quite active and new LSD are still being found. The present invention is intended to include those that are found from time to time as well as the categories of LSD selected from the group consisting of mucopolysaccharidases (MPSs), lipidoses, glycogenoses, oligosaccharidoses and neuronal ceroid lipofuscinoses. A listing of many of the LSD currently known and the defective enzymes are listed below in table A. It will be understood that the LSD listed therein are encompassed by the present invention.

Table A

Disease	**Clinical Phenotype**	**Enzyme Deficiency**
Aspartylglucosaminuria		Aspartylglucosaminidase
Cholesterol ester storage disease	Wolman disease	Acid lipase
Cystinosis		Cystine transporter
Fabry disease	Fabry disease	α-Galactosidase A

Farber Lipogranulomatosis	Farber disease	Acid ceramidase
Fucosidosis		α-L-Fucosidase
Galactosialidosis types I/II		Protective protein
Gaucher disease types I/II/III	Gaucher disease	Glucocerebrosidase (β-glucosidase)
Globoid cell leucodystrophy	Krabbe disease	Galactocerebrosidase
Glycogen storage disease II	Pompe disease	α-Glucosidase
GM1-Gangliosidosis types I/II/III		β-Galactosidase
GM2-Gangliosidosis type I	Tay Sachs disease	β-Hexosaminidase A
GM2-Gangliosidosis type II	Sandhoff disease	β-Hexosaminidase A & B
GM2-Gangliosidosis		GM2-activator deficiency
α-Mannosidosis types I/II		α-D-Mannosidase
β-Mannosidosis		β-D-Mannosidase
Metachromatic leucodystrophy		Arylsulphatase A
Metachromatic leucodystrophy		Saposin B
Mucolipidosis type I	Sialidosis types I/II	Neuramindase
Mucolipidosis types II/III	I-cell disease; pseudo-Hurler polydystrophy	Phosphotransferase
Mucolipidosis type IIIC	pseudo-Hurler polydystrophy	Phosphotransferase γ-subunit
Mucolipidosis type IV		Unknown
Mucopolysaccharidosis type I	Hurler syndrome; Scheie syndrome	α-L-Iduronidase
Mucopolysaccharidosis type II	Hunter syndrome	Iduronate-2-sulphatase
Mucopolysaccharidosis type IIIA	Sanfilippo syndrome	Heparan-N-sulphatase
Mucopolysaccharidosis type IIIB	Sanfilippo syndrome	α-N-Acetylglucosaminidase
Mucopolysaccharidosis type	Sanfilippo syndrome	AcetylCoA:N-acetyltransferase

IIIC		
Mucopolysaccharidosis type IIID	Sanfilippo syndrome	N-Acetylglucosamine 6-sulphatase
Mucopolysaccharidosis type IVA	Morquio syndrome	Galactose 6-sulphase
Mucopolysaccharidosis type IVB	Morquio syndrome	β-galactosidase
Mucopolysaccharidosis type VI	Maroteaux-Lamy syndrome	N-Acetylgalactosamine 4-sulphatase
Mucopolysaccharidosis type VII	Sly syndrome	β-Glucuronidase
Mucopolysaccharidosis type IX		hyaluronoglucosaminidase-1
Multiple sulphatase deficiency		Multiple sulphatases
Neuronal Ceroid Lipofuscinosis, CLN1	Batten disease	Palmitoyl protein thioesterase
Neuronal Ceroid Lipofuscinosis, CLN2	Batten disease	Tripeptidyl peptidase I
Neuronal Ceroid Lipofuscinosis, CLN3	Vogt-Spielmeyer disease	Unknown
Neuronal Ceroid Lipofuscinosis, CLN5	Batten disease	Unknown
Neuronal Ceroid Lipofuscinosis, CLN8	Northern Epilepsy	Unknown
Niemann-Pick disease types A/B	Niemann-Pick disease	Acid sphyngomyelinase
Niemann-Pick disease type C1	Niemann-Pick disease	Cholesterol trafficking
Niemann-Pick disease type C2	Niemann-Pick disease	Cholesterol trafficking
Pycnodysostosis		Cathepsin K
Schindler disease types I/II	Schindler disease	α-Galactosidase B
Sialic acid storage disease	Sialuria, Salla disease	Sialic acid transporter

The term "storage associated compound" use herein encompasses lipid containing primary storage material that accumulates in lysosomes of cells of the individual with the LSD concerned. The term storage associated compound also encompasses, lipid containing secondary material such as metabolites or catabolite of the primary storage material. The term storage associated material also encompasses lipid containing compounds the concentration of which alters as a consequence of the LSD such as might accumulates as a result of the proliferation of the membrane mass in the cells, or other secondary metabolic compounds that might for example decrease in level as a result of influence exerted by the increasing build up of primary storage material. The term is not intended to encompass the presence or absence of, for example, surface markers, specialised proteins such as enzymes or the like.

The estimated levels might refer directly to the principal storage compound and important candidates are secondary metabolites where these are lipid containing.

In certain forms of the invention the storage compounds might be very wide. They might include lipids and lipid containing macromolecules. The storage associated compounds might thus be selected from the group of compounds consisting of phospholipids and glycoconjugates

In forms where glycoconjugates are contemplated they might include glycolipids and lipopolysaccharides.

Glycolipids might be selected from the group comprising glycerolipids, glycoposhatidylinositols, glycosphingolipids. The glycosphingolipids might be selected from the group comprising neutral or acidic glycosphingolipids, monoglycosylceramides, or diosylcermaides, gangliosides, glycuronoglycosphingolipids, sulfatoglycosphingolipids, phosphoglycosphingolipids, phosphonoglycosphingolipids, sialoglycosphingolipids, uronoglycosphingolipids, sulfoglycosphingolipids, phosphoglycosphingolipids. Also contemplated may be sphinoglipids (including ceramide, glucosylceramide, trihexosylceramide), and globosides (including tetrahexosylceramides).

The phospholipid useful for the present invention is not intended to be limited. Phospholipids encompassed by the invention might be characterised by their head groups which might be selected from, but not limited to, the group consisting of phosphatidyl serine, phosphatidylinositol, phosphatidyl ethanolamine and sphingomyelin phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, phosophatidyl ethanolamine, cerebroside or a ganglioside.

The phospholipids might be characterised by the fatty acids which might be selected from, but not limited to, the group consisting of 1-palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl -, 1-palmitoly-2-arachadonyl -, 1-palmitoyl-2-docosahexanoyl. However other fatty acyl groups might also be chosen and could be selected from those having acyl chains of about 12 to about 18 carbon atoms. These tail group will be understood to be combined with any one of the head groups of the immediately preceding paragraph.

The method of measuring the presence and relative levels of storage associated compounds is not important to the general approach of the invention, and might be selected from any convenient method. Such methods might include electrophoresis, chromatography, Gas chromatography, HPLC (High pressure Liquid Chromatography), Nuclear Magnetic resonance analysis, gas chromatography-mass spectrometry (GC-MS), GC linked to Fourier-transform infrared spectroscopy (FTIR), and silver ion and reversed-phase high-performance liquid chromatography (HPLC) as wells as mass spectrometry.

As the complex relationships between stored substrates and pathology in LSD become clearer there is an obvious advantage of providing for faster and more accurate methods to characterise and quantify these stored substrates. That is particularly the case where the storage associated compounds needs to be measured in complex biological samples such as urine, plasma, and blood. To that end it is preferred to use mass spectrometry. The type of mass spectrometry method selected from the group consisting of ionising mass spectrometry, quadrupole mass spectrometry, ion trap mass spectrometry, time-of-

flight mass spectrometry and tandem mass spectrometry, and electrospray ionization (ESI), the later being considered advantageous.

Particularly advantageous is electrospray ionisation-tandem mass spectrometry (ESI-MSMS). The advent of electrospray ionisation-tandem mass spectrometry (ESI-MSMS) has made possible the simultaneous determination of large numbers of analytes from complex mixtures. For newborn screening, ESI MSMS enables the concurrent determination of a wide range of amino acids and acyl carnitines as their butyl esters. This technology is used to screen for over twenty different genetic disorders, including the amino acidopathies and the fatty acid oxidation defects (6,7). ESI-MSMS has been used effectively to investigate stored substrates in a number of LSD and has great potential in the field of this invention.

It has become evident that the levels of a single storage associated compound are not sufficient to give a clear distinction between varying degrees of exposure of an individual to the effects of an LSD. A comparison between at least two markers is required for a quantitative relationship to emerge. The relationship might be additive so that both storage associated compounds increase in the levels in which they are found where the condition is present, and a comparison is made to an internal control. Preferably in devising the method where at least two compounds are selected one from a first group that increase and a second from a second group that decreases in levels. The values are combined mathematically to arrive at an index number. The relative levels of those two compounds leads to an amplification of the differences between LSD affected individuals and the control population. As indicated earlier the severity of the condition and the index number have a direct correlation. Conversely therefore the value of the index number can be compared to a standard to provide a indication of the level of severity of the condition.

It has been found that a difference in index number between individuals that are positive or negative for an LSD condition by use of such combination can be made statistically significant provided an appropriate combination of storage associated compounds is used.

Samples for analysis can be obtained from any organ, tissue, fluid or other biological sample comprising lysosomes or their component storage associated compounds. A preferred sample is whole blood and products derived therefrom, such as plasma and serum. Blood samples may conveniently be obtained from blood-spot taken from, for example, a Guthrie card.

Other sources of tissue for example are skin, hair, urine, oral fluids, semen, faeces, sweat, milk, amniotic fluid, liver, heart, muscle, kidney, brain and other body organs. Tissue samples comprising whole cells are typically lysed to release the storage associated compounds.

The present method may be used as an early test and thus samples can be obtained from embryos, foetuses, neonatals, young infants.

Most preferably the sample is one readily obtainable such as a blood samples. Whilst obtaining these is invasive they are routinely taken and generally therefore are not inconvenient. It may be preferred to have a non-invasive sample such as urine, oral fluid or buccal smear. There are however variations in the value of certain metabolites in urine resulting from variation in salt content, such as oxalic acid, and in saliva there is variation in the capacity of individuals to secrete certain compounds.

It is found that with Gaucher patients that the LSD index number was not only a qualitative measure but also a qualitative measure being indicative of the severity of the condition. Thus the status of the LSD being assessed may not only be to ascertain the presence or absence but might also include the degree of severity. The status might also include subclinical levels of the condition that relate to levels achieved before onset of physical manifestations become apparent. This invention will be understood to have application to monitoring treatment, for example with individuals undergoing enzyme or other therapy.

Thus individuals with Gaucher disease that undergo enzyme replacement therapy have a index number that is considerably lower than untreated individuals. It is also desirable that the doses of active enzyme delivered to sufferers is kept to a minimum if only from a cost perspective but perhaps also from a perspective of minimising any adverse affects of the treatment. Thus the present method may be used particularly for monitoring treatment of an LSD sufferer, or for ascertaining initially and perhaps from time to time as the sufferer ages the most appropriate dose of active to be delivered, and thus individuals diagnosed may be tested from time to time to ascertain the severity of the condition. It is less critical that the test discriminates quite as distinctly from non-LSD sufferers because all that is required is that the relative level of severity can be quantified. Thus whilst it may be necessary to screen using indicators of the concentration of three or more lipid containing compounds to distinguish over non-LSD sufferers the monitoring may only require indicators of two lipid containing compounds and may be carried out using less precise measuring methods.

The invention has particular applicability to human conditions. Certain mammals are also susceptible to LSD and the invention may be useful where the individual is a non-human mammal. For examples α-mannosidoses is relatively common in certain breeds of cattle and screening may be a useful stock management tool.

EXAMPLE 1 MONITORING OF THERAPY FOR GAUCHER DISEASE

This report provides a detailed analysis of the initial trial of our developed methodology to monitor enzyme replacement therapy (ERT) in Gaucher disease using dried blood spots.

Patient samples: Dried blood spots have been collected from five Australian Gaucher patients receiving ERT for the past two years (12 samples). Sixteen dried blood spots have been collected from patients not receiving ERT, from referrals to the National Referral Laboratory for Lysosomal, Peroxisomal and Related Diseases (which is based in our parent Department). In addition, through collaboration with Dr Eugene Mengel (Germany), we have obtained 39 samples from German Gaucher disease patients receiving ERT, and three samples from untreated patients. Dried blood spots have been

collected from 10 unaffected adults as control samples. Total sample numbers are as shown in Table 1.

Sample preparation: From each Guthrie card sample a 3 mm dried blood spot was punched and the lipids were eluted (16h) with 200 μL of isopropanol containing 200 nmol of each internal standard; Cer C17:0, GC(*d3*)C16:0, LC(*d3*)C16:0, PC C14:0. The blood spots were removed and the isopropanol dried under a stream of nitrogen. Lipids were redissolved in 100 μL of methanol containing 10 mM NH_4COOH for analysis by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 μL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2 x 10^{-5} Torr. Lipids were analysed in +ve ion mode. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species were monitored using the ion pairs shown in Table 2. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Table 2).

RESULTS

To determine which analytes were potentially useful markers for monitoring Gaucher disease, the patients were grouped into control (group 1, n=10), Gaucher patients receiving ERT (group 2, n=51), and untreated Gaucher patients (group 3, n=19). Mann-Whitney U values were then calculated for each analyte to determine the difference between the control and untreated patients, control and treated patients, and treated and untreated patients. These results are shown in Table 3.

We observed that, in addition to the expected elevation of glucosylceramide (GC) in the untreated Gaucher patients compared to controls, there were significant differences in the level of ceramide C16:0 and the sphingomyelin species C16:0, C22:0 and C24:0 (all significant to the 0.01 level). The same markers also showed a significant difference between treated and untreated Gaucher patients. Of the lactosylceramide and trihexosylceramide species only the C16:0 species showed a significant difference between control and untreated patients (significant to the 0.05 level). The box plots of each C16:0 species of ceramide, GC, LC and sphingomyelin (Figures 1 and 2) show that whilst there is an observed increase in the level of ceramide and GC in untreated patients, the levels of sphingomyelin and LC are decreased. In addition, the level of these analytes in the treated patients generally fell between the control and untreated patients. In each case ERT has partially normalised the lipid levels, although not in all patients.

Although the observed differences between control and untreated patients are significant there is still considerable overlap between the two populations. This is due, at least in part, to the range of lipid levels in the control and patient groups. To improve the discrimination of the markers we investigated the use of multiple markers by plotting ratios of GC/LC or ceramide/sphingomyelin (Figure 3). As GC and ceramide levels increase in Gaucher patients, while the LC and sphingomyelin decrease, these ratios provided improved discrimination between groups. Utilising all four analytes in a combined ratio (Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0) further improved the discrimination. Similarly discriminate analysis using the four C16:0 species resulted in a function (Dis2 = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91)) with improved discrimination. (Figure 4 and Table 3).

Clearly, the use of multiple analytes or lipid profiles provides a better representation of lipid metabolism in control and Gaucher patients. The ratio4 and discriminate function (Dis2) plotted in Figure 4 show almost total separation of the control and untreated Gaucher patient groups, with the patient group being partially normalised (although many treated patients were not completely normalised).

We investigated what effect time on therapy had on a number of the same analytes and analyte ratios (Figure 5 and 6). The GC and ceramide levels showed a trend towards normalisation with increasing time on therapy, however in a number of patients the ceramide level did not reach the normal range even at 80-120 months on therapy. The use of the ratio and the discriminate function (Figure 6) showed similar results with some patients normalising with time but others outside the normal range even after 80-120 months of therapy.

The relationship between the glycolipid markers and ratios, and the macrophage activation marker chitotriosidase is shown in Figures 7-9; a significant correlation is observed for the ceramide and GC as well as for the ratios GC/LC, ceramide/sphingomyelin and ratio4, and for the discriminate function. Table 4 shows the Pearson correlation coefficients for these markers with chitotriosidase and other markers that have been used to monitor ERT in Gaucher disease including angiotensin converting enzyme, lysozyme and acid phosphatase. In general the correlations are stronger between these markers and the lipid ratios, rather than single lipid species.

DISCUSSION

In this study we have provided evidence that the primary storage substrate GC is a useful marker for monitoring Gaucher disease. We observe an increased level of GC in dried blood spots from untreated patients compared to controls and a normalisation of GC levels after ERT. This is an expected outcome, based on the known biochemistry of Gaucher disease. Somewhat less expected is the elevation in ceramide and the decrease in LC and sphingomyelin. We have previously reported that LC is decreased in the plasma of Gaucher patients and that the ratio of GC/LC provides a better discrimination of Gaucher patients from controls than the GC levels on their own (Whitfield et al 2002). In these preliminary studies we have identified that other lipids are also affected, particularly ceramide and sphingomyelin. We have also shown that using a combination of these analytes with the GC and LC levels, as either a ratio or a discriminate function, provides greater discrimination and potentially a better mechanism for monitoring ERT in Gaucher disease than the use of individual analytes.

The ratio4 and the discriminate function Dis2 are based on the limited numbers in this study and require further refinement, however they provide an initial demonstration of the power of metabolic profiling for the characterisation of patients and the monitoring of therapy in Gaucher disease.

Our hypothesis is that the level of GC within a normal population will fall within a specified range, which is affected by many metabolic parameters affecting the biosynthesis and degradation of GC. In the Gaucher disease population this range will be altered as a result of the metabolic defect; however, those Gaucher patients with the lower GC levels are likely to overlap with unaffected controls with the higher GC levels. This results in uncertainties in the interpretation of GC levels in isolation with regard to Gaucher disease status, and difficulties in determining normalisation following ERT.

However, with ametabolic profile (multiple analytes) the breadth of the normal range will be decreased, as each of these analytes is related to the others by the metabolic pathways that exist. Consequently, the power to discriminate normal from Gaucher disease is increased and the ability to measure the normalisation of patients on treatment is improved.

Table 1. Patient and control samples included in this trial

Patient group	Number	Age Median (range)	Comment
Control	10	38 (23-56)	
Treated Gaucher	51	23 (2-72)	All type 1
Untreated Gaucher	19	24 (1-36)	2 type 3, 14 type 1, 3 unknown

Table 2. Lipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

Table 3. Mann-Whitney U values for lipid analytes and ratios of analytes, between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

	Control vs Untreated		Control vs Treated		Untreated vs Treated	
Analyte	M-W U[d]	Sig.[e]	M-W U[d]	Sig.[e]	M-W U[d]	Sig.[e]
Cer C16:0	6	0.000	111	0.004	300	0.009
Cer C24:1	73	0.313	215	0.342	478	0.740
Cer C24:0	56	0.070	174	0.087	447	0.466
GC C16:0	9	0.000	139	0.017	240	0.001
GC C22:0	26	0.002	142	0.021	307	0.012
GC C24:1	19	0.000	101	0.002	271	0.003
GC C24:0	28	0.002	149	0.029	319	0.018
LC C16:0	49	0.033	222	0.419	358	0.063
LC C24:0	75	0.359	183	0.121	450	0.490
LC C24:1	62	0.130	228	0.481	434	0.375
CTH C16:0	52	0.046	149	0.028	392	0.152
CTH C22:0	83	0.582	127	0.009	166	0.000
CTH C24:1	88	0.748	103	0.002	189	0.000
CTH C24:0	54	0.060	179	0.104	472	0.687
SM C16:0	31	0.003	239	0.618	149	0.000
SM C22:0	29	0.002	203	0.240	187	0.000
SM C24:0	33	0.004	219	0.382	219	0.000
GC_LC	6	0.000	80	0.001	169	0.000
CER_SM	9	0.000	150	0.031	138	0.000
RATIO4[f]	7	0.000	64	0.000	96	0.000
DIS2[g]	9	0.000	164	0.057	86	0.000

[a] controls n=10

[b] untreated n= 19

[c] treated n= 51

[d] Mann-Whitney U values

[e] significance (two-tailed)

[f] Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0)

[g] Dis2 = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91

Table 4. Pearson Correlation coefficients between lipid markers and other markers used in Gaucher disease.

Analyte[a]	months of therapy[b]		chitotriosidase(nmol/ml/h)		ACE (U/l)[e]		lysozyme (mg/l)		acid phosphatase	
	PCC[c] N=51	Sig.[d]	PCC N=30	Sig.	PCC N=40	Sig.	PCC N=38	Sig.	PCC N=40	Sig.
Cer C16:0	-0.24	0.08	0.40	0.03	0.42	0.01	0.40	0.01	0.44	0.00
GC C16:0	-0.32	0.02	0.41	0.02	0.36	0.02	0.23	0.17	0.52	0.00
LC C16:0	0.19	0.18	0.16	0.38	0.10	0.53	0.01	0.96	0.17	0.30
CTH C16:0	0.00	1.00	-0.10	0.60	-0.03	0.83	0.34	0.04	-0.01	0.95
SM C16:0	0.51	0.00	-0.29	0.13	-0.24	0.13	0.04	0.82	-0.23	0.15
GC/LC	-0.35	0.01	0.42	0.02	0.41	0.01	0.23	0.17	0.50	0.00
CER/SM	-0.47	0.00	0.52	0.00	0.50	0.00	0.35	0.03	0.53	0.00
RATIO4	-0.38	0.01	0.59	0.00	0.58	0.00	0.39	0.01	0.70	0.00
DIS2	0.54	0.00	-0.49	0.01	-0.47	0.00	-0.26	0.11	-0.47	0.00

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihexoside, SM = sphingomyelin, Ratio4 = (GC C16:0*Cer C16:0)/(LC C16:0*SM C16:0), Dis = (-195*Cer C16:0) - (29.8*GC C16:0) + (12.3*LC C16:0) + (16.9*SM C16:0) - 1.91

[b] months on enzyme replacement therapy

[c] PCC = Pearson correlation coefficient

[d] Sig. = significance (two tailed)

[e] ACE = angiotensin converting enzyme

EXAMPLE 2.

IDENTIFICATION OF FABRY HEMIZYGOUS AND HETEROZYGOUS INDIVIDUALS USING LIPID PROFILES.

This report summarises the results of analyses performed on urine, plasma and dried blood spots from control, Fabry heterozygote and Fabry patient groups.

MATERIALS AND METHODS

Patient samples: Urine samples have been collected from 14 Fabry patients (two of whom had renal transplants), 13 Fabry heterozygotes (three of whom had reported clinical symptoms) and 20 unaffected controls. Plasma samples were retrieved from archival sources in the Department of Chemical Pathology and represented 29 Fabry patients, three Fabry heterozygotes and 10 control samples. Dried blood spots on filter

paper (Guthrie cards) were collected from 13 Fabry patients, two Fabry heterozygotes and 10 control individuals.

Sample preparation and analysis: Urine, plasma and dried blood spot samples were prepared as described in Appendices I, II and III, and analysed for lipids by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 μL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2 x 10^{-5} Torr. Lipids were analysed in +ve ion mode. Lipid analysis was performed using the multiple-reaction monitoring (MRM) mode. Twenty-two different ceramide, glycosphingolipid and sphingomyelin species were monitored using the ion pairs shown in Table 5. In urine samples seven additional phosphatidylcholine species were also monitored (Table 5). Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Table 5).

RESULTS

Analysis of Urine: Lipid profiling of the urine samples from control, Fabry and Fabry heterozygotes (Fabry het) has been performed. In all, 29 lipid species were determined including ceramide (Cer), glucosylceramide (GC), lactosylceramide (LC), trihexosylceramide (CTH), sphingomyelin (SM) and phosphatidylcholine (PC) species. Appropriate internal standards were used that provide absolute quantification of these species (expressed as nmol/L urine). PC was included as a general marker of urinary sediment, as we had previously observed this to be a more useful correction factor for the determination of urinary lipids than creatinine. This relates to the urinary lipids being derived from epithelial cells of the kidneys, bladder and urinary tract rather than

filtered through the kidneys; PC is a major lipid constituent of these cells and so is a useful measure of the level of urinary sediment.

An initial statistical analysis was performed on the data as expressed as nmol/L urine. Mann-Whitney U values were determined to compare the control group with the Fabry and Fabry het groups (Table 6). Examination of these results shows that many of the lipid analytes are significantly different in the patient groups compared to the control groups. The Fabry and Fabry het groups show a significant difference to the control group in many lipid species, including Cer, LC, CTH and SM. Interestingly, the level of PC in the Fabry het group is significantly elevated above the control population, while no significant difference between the control and Fabry groups is observed. Examination of the range of analytes for each group (Figure 10) shows that for all analytes except CTH, the Fabry het group is elevated above the control and Fabry groups. The observed elevation of these lipids suggests that the Fabry het group has elevated urinary sediment compared to the control and Fabry groups.

The scatter plot of LC (total) versus CTH (total) (Figure 11A) shows that the use of lipid levels (nmol/L urine) can differentiate between Fabry patients and the control group, although there is some overlap between both Fabry and Fabry het and the control group. The use of the specific lipid species LC C24:1 and CTH C24:1 (Figure 11B) improved this discrimination, although some overlap still exists. A concern with these results is that the differentiation of the Fabry het group from the control group reflects the elevated urinary sediment rather than an altered lipid profile. Consequently, individuals who are not affected by Fabry disease but who have an elevation in urinary sediment would be falsely identified as a Fabry het using this type of analysis.

To address this, correction was made for each lipid analyte value for the level of PC (total) in each sample; statistical analysis on these data was performed. Table 7 shows the Mann-Whitney U values for each patient group compared to the control group. The corrected data also show multiple analytes to be significantly different between the control and patient groups. The box plots in Figure 12 show the range of each analyte group (corrected for PC). These plots show that the Fabry group has elevated CTH, LC

and Cer and decreased SM, whereas the Fabry het group now shows an elevation in CTH and a much lower elevation in LC and Cer. Interestingly, the Fabry het group shows a larger decrease in the SM than the Fabry group. This may relate to a sex difference, although no difference was seen between the males and females in the control group. Larger sample numbers will be required to confirm this.

As with the urine data expressed as nmol/L the differentiation between control and patient groups could be improved by the selection of specific lipid species. The increases observed in Cer, LC and CTH were greatest in the C24:1 species, and the decreases observed in GC and SM were greatest in the C24:0 species (Figure 13). Following these observations we looked at the relationship between these lipid species in a series of scatter plots and how these were able to differentiate the control and patient groups (Figure 14). Using different combinations we can achieve almost total differentiation between the control and patient groups, particularly with CTH C24:1 and LC C24:1 plotted as a function of SM C24:0 (Figures 5D and 5E).

LC and CTH are elevated while GC and SM are decreased in the patient groups. The use of ratios of these analytes enables further discrimination between the control and patient groups. Figure 15 shows total separation of both Fabry and Fabry het groups from the control group.

Of interest is the observation that the composition of individual PC species is significantly altered in the Fabry group compared to the control group. Some PC species show a proportional elevation (C34:2 and C36:4) while others show a corresponding decrease (C32:1 and C34:1) (Figure 16). On first examination there appears to be a trend toward higher levels of unsaturated fatty acids in the Fabry group. This is supported by the observation that the LC C24:1 and CTH C24:1 species show a greater elevation in the Fabry group compared to the C24:0 species. The effect of these changes in the lipid composition to the cellular function in Fabry disease and the relationship to the pathophysiology of this disorder is unclear at this time. However, we are further investigating these effects in cultured skin fibroblasts from control and Fabry patients. Results will be available in subsequent Reports.

To summarise, analysis of the lipid profile in urine from control, Fabry and Fabry het groups has identified the specific lipid species, ratios and profiles that best discriminate between the control and patient groups. Correction of the lipid species for PC content of the urine improved the discrimination between control and Fabry groups and minimised the potential for the false identification of individuals with high urinary sediment as Fabry hets. The "Ratio 4" (LC C24:1*CTH C24:1)/(GC C24:0*SM C24:0) provides total discrimination of all Fabry and Fabry hets from the control group.

Analysis of Plasma: The number of plasma and blood spot samples available from the Fabry het group were fewer than the urine samples. However, lipid profiles were performed on these samples and the Mann-Whitney U values for each lipid species are shown in Table 8. No significant difference is observed between the control and Fabry het groups (possibly due to the low number of Fabry het samples), however Cer, LC, CTH and SM species show significant differences between the control and Fabry groups. Figure 17 shows that Cer, LC and SM are decreased in the Fabry group compared to the control group, while CTH is increased and GC is unchanged, although it did appear to have a broader range in the Fabry group. When the Cer, GC, LC and SM C16:0 species were plotted as a function of the CTH C16:0 (Figure 18) a strong correlation is observed in the Fabry group, which provides improved discrimination between the control and Fabry groups.

Analysis of Whole Blood: Analysis of dried blood spots for lipids show relatively few analytes with significant differences between the control and Fabry groups (Table 9). Box plots of the lipid groups (Figure 19) show only slight elevations or decreases in the Fabry compared to the control groups, and only the CTH has a p value of less than 0.05. The use of specific lipid species offers little improvement although the decrease of Cer C24:1 in the Fabry group compared to the control group is significant (p= 0.03) (Figure 20). The box plots of the CTH species show that only the C16:0, C18:0 and C20:0 species are significantly different from the control group (Figure 21 and Table 9). The scatter plot of CTH C16:0 as a function of Cer C16:0 (Figure 22A) shows a similar correlation between these two analytes, as was observed in the plasma samples. The

correlation is not as pronounced in the plot of CTH C18:0 as a function of SM C16:0 (Figure 22B). The Fabry het group did not show any significant difference to the control group in the lipid analytes.

DISCUSSION

The use of a urinary lipid profile also has potential to identify Fabry and Fabry heterozygotes. While the determination of CTH on its own did not identify all patients, the use of ratios of lipid species provided total discrimination of both the Fabry patients (even after renal transplant) and the heterozygotes from the control group. Urine analysis is a practical, non-invasive procedure to screen large populations at high risk for Fabry disease.

Monitoring of therapy: Characterisation of the lipid profile of Fabry patients in plasma, dried blood spots and urine has highlighted a number of previously unreported differences between Fabry patients and the control population. This technology enables us to very accurately describe the lipid profile from the control population and so define how the profile differs in Fabry disease. Significant differences were observed in most lipid groups suggesting that Fabry disease results in a general alteration of lipid metabolism, not just the storage of trihexosylceramide. With further validation it will be possible to monitor therapy in Fabry disease by following the total lipid profile as it is corrected from the disease state to a normal profile. This will provide a more comprehensive Fabry monitoring program than current methods allow. We are currently investigating the potential of this approach with patient samples and cultured skin fibroblasts.

Prediction of disease severity: The detailed description of the disease state provided by the lipid profile described in this Report will significantly improve our ability to describe the disease in any given individual. Correlation of these profiles with known phenotypes and disease progression will enable us to predict disease progression.

Table 5. Lipid analytes used for lipid analysis of Fabry samples

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C20:0	LC(*d3*)C16:0	918.6/264.4
LCC22:0	LC(*d3*)C16:0	946.7/264.4
LC C22:0-OH	LC(*d3*)C16:0	962.7/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
CTH C16:0	CTH C17:0	1024.1/264.4
CTH C18:0	CTH C17:0	1052.1/264.4
CTH C20:0	CTH C17:0	1080.1/264.4
CTH C22:0	CTH C17:0	1108.1/264.4
CTH C24:0	CTH C17:0	1136.6/264.4
CTH C24:1	CTH C17:0	1134.1/264.4
CTH C17:0 (internal standard)		1038.1/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C32:0	PC C14:0	706.5/184.1
PC C32:1	PC C14:0	704.5/184.1
PC C34:1	PC C14:0	732.5/184.1
PC C34:2	PC C14:0	730.5/184.1
PC 36:2	PC C14:0	758.6/184.1
PC C36:4	PC C14:0	754.6/184.1
PC C38:4	PC C14:0	782.6/184.1
PC C14:0[b] (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

[b] PC C14:0 is a commercial standard and is known to have a C16:0 second fatty acid (equivalent to PC C30:0)

Table 6 Mann-Whitney U values for lipid[a] analytes in urine.

Analyte[b]	Control (n=20) vs Heterozygote (n=13)		Control (n=20) vs Fabry (n=14)	
	MW-U	p value	MW-U	p value
Cer C16:0	41	0.000	81	0.018
Cer C24:0	82	0.037	118	0.243
Cer C24:1	62	0.006	68	0.005
GC C16:0	63	0.006	144	0.746
GC C22:0	87	0.056	119	0.256
GC C24:0	69	0.012	118	0.243
GC C24:1	71	0.014	153	0.974
LC C16:0	18	0.000	61	0.003
LC C20:0	41	0.000	56	0.001
LCC22:0	34	0.000	77	0.012
LC C22:0-OH	37	0.000	81	0.018
LC C24:0	23	0.000	17	0.000
LC C24:1	11	0.000	2	0.000
CTH C16:0	3	0.000	56	0.001
CTH C18:0	61	0.005	46	0.000
CTH C20:0	22	0.000	59	0.001
CTH C22:0	2	0.000	43	0.000
CTH C24:0	4	0.000	37	0.000
CTH C24:1	0	0.000	25	0.000
SM C16:0	80	0.031	115	0.206
SM C22:0	83	0.041	74	0.009
SM C24:0	120	0.432	70	0.006
PC C32:0	50	0.001	146	0.795
PC C32:1	56	0.003	94	0.052
PC C34:1	65	0.008	129	0.417
PC C34:2	63	0.006	109	0.144
PC 36:2	61	0.005	148	0.846
PC C36:4	64	0.007	103	0.098
PC C38:4	74	0.018	126	0.364
Cer (total)	56	0.003	109	0.083
GC (total)	64	0.007	137	0.386
LC (total)	14	0.000	40	0.000
CTH (total)	0	0.000	37	0.000
SM (total)	85	0.048	84	0.012
PC (total)	62	0.006	164	0.975

[a] lipids expressed as nmol/L urine.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin, PC = phosphatidylcholine.

Table 7 Mann-Whitney U values for lipid[a] analytes in urine.
(Corrected for phospatidylcholine content)

Analyte[b]	Control (n=20) vs Heterozygote (n=13)		Control (n=20) vs Fabry (n=14)	
	MW-U	p value	MW-U	p value
Cer C16:0	95	0.101	73	0.009
Cer C24:0	90	0.070	107	0.127
Cer C24:1	141	0.946	60	0.002
GC C16:0	133	0.733	152	0.948
GC C22:0	62	0.006	109	0.144
GC C24:0	63	0.006	119	0.256
GC C24:1	89	0.065	148	0.846
LC C16:0	37	0.000	63	0.003
LC C20:0	128	0.609	69	0.006
LCC22:0	107	0.219	80	0.016
LC C22:0-OH	125	0.539	71	0.007
LC C24:0	62	0.006	2	0.000
LC C24:1	34	0.000	2	0.000
CTH C16:0	87	0.056	35	0.000
CTH C18:0	126	0.562	33	0.000
CTH C20:0	128	0.609	35	0.000
CTH C22:0	68	0.010	26	0.000
CTH C24:0	78	0.026	11	0.000
CTH C24:1	43	0.001	4	0.000
SM C16:0	42	0.001	70	0.006
SM C22:0	47	0.001	0	0.000
SM C24:0	43	0.001	4	0.000
PC C32:0	120	0.432	83	0.021
PC C32:1	136	0.811	28	0.000
PC C34:1	72	0.015	39	0.000
PC C34:2	143	1.000	31	0.000
PC 36:2	84	0.044	135	0.538
PC C36:4	127	0.585	20	0.000
PC C38:4	75	0.020	93	0.048
Cer (total)	119	0.413	82	0.019
GC (total)	83	0.041	129	0.417
LC (total)	77	0.024	26	0.000
CTH (total)	97	0.116	19	0.000
SM (total)	42	0.001	12	0.000

[a] lipids expressed as nmol/umol PC.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin, PC = phosphatidylcholine.

Table 8. Mann-Whitney U values for lipid[a] analytes in plasma.

Analyte[b]	Control (n=10) vs Heterozygote (n=2)		Control (n=10) vs Fabry (n=29)	
	MW-U	p value	MW-U	p value
Cer C16:0	9	0.830	59	0.007
Cer C24:1	7	0.519	34	0.000
Cer C24:0	9	0.830	48	0.002
GC C16:0	9	0.830	136.5	0.908
GC C22:0	9	0.830	134	0.842
GC C24:1	6	0.390	137.5	0.934
GC C24:0	2	0.085	124	0.596
LC C16:0	9	0.830	66	0.014
LC C24:1	8	0.667	33	0.000
LC C24:0	4	0.197	4.5	0.000
CTH C16:0	8	0.667	33	0.000
CTH C18:0	7	0.519	19.5	0.000
CTH C20:0	9	0.830	49	0.003
CTH C22:0	4	0.197	45	0.002
CTH C24:1	10	1.000	49	0.003
CTH C24:0	8	0.667	53	0.004
SM C16:0	10	1.000	33	0.000
SM C22:0	4	0.197	39	0.001
SM C24:0	8	0.667	29	0.000
Cer (total)	7	0.519	38.5	0.001
GC (total)	5	0.282	138	0.947
LC (total)	8	0.667	48	0.002
CTH (total)	10	1.000	38	0.001
SM (total)	8	0.667	37	0.001

[a] lipids were calculated as umol/L plasma.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin.

Table 9: Mann-Whitney U values for lipid[a] analytes in whole blood.

Analyte[b]	Control (n=10) vs Heterozygote (n=2)		Control (n=10) vs Fabry (n=13)	
	MW-U	p value	MW-U	p value
Cer C16:0	8	0.235	48	0.292
Cer C24:1	8	0.237	30	0.030
Cer C24:0	12	0.612	46.5	0.251
GC C16:0	14	0.866	39	0.107
GC C22:0	7.5	0.202	40.5	0.128
GC C24:1	15	1.000	47.5	0.278
GC C24:0	9	0.310	38.5	0.100
LC C16:0	13	0.735	37.5	0.088
LC C24:1	7	0.175	61.5	0.828
LC C24:0	12	0.612	40.5	0.129
CTH C16:0	10	0.398	6	0.000
CTH C18:0	8	0.237	42.5	0.163
CTH C20:0	9	0.310	45	0.215
CTH C22:0	10	0.398	40	0.121
CTH C24:1	7.5	0.204	1.5	0.000
CTH C24:0	6	0.128	32	0.041
SM C16:0	7.5	0.204	53.5	0.475
SM C22:0	9	0.310	61	0.804
SM C24:0	11	0.499	55.5	0.556
Cer (total)	9	0.310	38	0.094
GC (total)	13	0.735	37	0.082
LC (total)	11	0.499	42	0.154
CTH (total)	9	0.310	23	0.009
SM (total)	12	0.612	63.5	0.926

[a] lipids were calculated as umol/L plasma.

[b] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = trihexosylceramide, SM = sphingomyelin.

APPENDIX I: Procedure for sphingolipid extraction from urine (Bligh-Dyer method).

1. To 1.5 mL urine add 5.6 mL $CHCl_3$/MeOH (1:2)
2. Add 400 pmol internal standards to each sample; 2 μL (d3) C16:0 LC (200 μM); 2 μL (d3) C16:0 GC (200 μM), and 2 μL GM2 (200 μM), 6.25 μL CTH C17:0 (64 μM); 2 μL Cer C17:0 (200 μM), 2 μL PC (200 μM).
3. Place tubes on platform shaker for 10 minutes at 150 opm. Stand tubes at room temperature for at least 50 minutes.
4. Partition with the addition of 1.9 mL $CHCl_3$ and 1.9 mL milliQ H_2O or KCl.
5. Place tubes on platform shaker for 10 minutes at 150 opm.
6. Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.
7. Wash the lower phase with the addition of 0.5 mL of B&D synthetic upper phase and vortexing briefly.
8. Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.
9. Dry samples (lower phase) under N_2 at 40°C (add water to heating block around tube to aid in evaporation). Periodically vortex the samples during the drying down process to ensure the highest recovery possible.
10. Resuspend extracts in 150 μL of MeOH containing 10 mM ammonium formate.

APPENDIX II: Procedure for glycolipid, phospholipid and ganglioside extraction from plasma (Folch extraction).

1. Add 100 μL plasma to a 12 mL glass tube with black screw cap lid.
2. Add 2 mL $CHCl_3$/MeOH (2:1) (at least 20 volumes of $CHCl_3$/MeOH to each sample).
3. Add internal standards to each sample 2 μL (d3) C16:0 LC (200 μM); 2μL (d3) C16:0 GC (200 μM), and 2 μL GM2 (200 μM), 6.25 μL CTH C17:0 (64 μM); 2 μL Cer C17:0 (200 μM); 2 μL PC (200 μM).

4. Shake for 10 minutes at 150 rpm. Stand on the bench at room temperature for at least 50 minutes.

5. Partition with the addition of 0.2 volumes (ie. 0.4 mL) of milliQ H_2O and vortex.

6. Centrifuge at 4000 x g for 10 minutes then gently remove upper aqueous layer, transferring it to a clean glass tube with a glass pipette for use in the ganglioside extraction and set aside (*refer to ganglioside extraction*). Carefully remove and discard the protein interphase.

7. Dry samples (lower phase) under N_2 at 40°C.

8. Resuspend samples in 20 µL methanol and add 0.18 mL $CHCl_3$ (containing 1% ethanol) and vortex to ensure sample is resuspended.

9. Pre-wash silica reverse phase columns (100 mg) with 3 mL acetone/methanol (9:1) followed by 3 mL $CHCl_3$ (containing 1% ethanol).

10. Load sample with a glass pipette and allow it to completely enter the solid phase, then wash with 3 mL $CHCl_3$ (containing 1% ethanol) (neutral lipids (ceramide) will go through and glycolipids/phospholipids will bind to the column).

11. Elute the glycolipids and phospholipids from the column into a clean 12 mL glass tube with black screw cap lid with 3 mL acetone/methanol (9:1) and vacuum dry columns briefly. (LC and GC internal standards are present in this fraction.)

12. Elute the phospholipids from the column into clean 12 mL glass tube with black screw cap lid with 3 mL methanol and vacuum dry columns briefly. (PC internal standard is present in this fraction if used.)

Note: Omitting step 10 will result in the glycolipids and phospholipids being eluted together.

13. Dry samples under N_2 at 40°C

14. Resuspend samples in 100 µL MeOH and store at –20°C.

15. Prior to running on the mass spectrometer resuspend samples into a final volume of 200 µL methanol containing 10 mM ammonium formate.

Ganglioside extraction

1. Follow glycolipid and phospholipid extraction procedure to step 6, taking upper aqueous phase from Folch extraction following H_2O partition.
2. Prime 25 mg C18 columns with 3 x 1 mL MeOH, followed by 3x1 mL MQ water.
3. Load upper phase to column with a glass pipette and allow solution to completely enter the solid phase of the column, then wash with 3 x 1 mL MQ water.
4. Elute gangliosides from the column into a clean 12 mL glass tube with black screw cap lid with 2 x 1 mL MeOH and vacuum dry columns briefly.
5. Dry samples under N_2 at 40^0C
6. Store samples at -20^0C.
7. Prior to running on the mass spectrometer resuspend in 200 µL methanol containing 10 mM ammonium formate.

APPENDIX III: Procedure for Extraction of Glycosphingolipids from Guthrie Spots

Materials and Reagents:

Isopropanol standards mixture:

1.0 µM Phosphatidylcholine C14:0/C14:0 (MW=678)

1.0 µM Glucosylceramide(d3) C18:0 (MW=703.8)

1.0 µM Lactosylceramide(d3) C16:0 (MW=865.6)

1.0 µM Ceramide C17:0 (MW=252.7)

1.0 µM Tri-hexose ceramide CTH C17:0 (MW=1038.9)

1.0 µM Monosialoganglioside GM2 (MW=1384.9)

1 x 1 mL 96 deep-well, v-bottom tray (polypropylene) and lid

1 x 250 µL v-bottom tray

Multichannel pipette

Plate-shaker

Experimental Procedure:

1. Place two 3 mm blood spots per sample in each well of a 96 deep-well, v-bottom tray.

2. Add 200 µL isopropanol containing standards (200 pmol of each standard) to each sample.
3. Cover tray with polypropylene plastic lid and shake samples for 2 hours on amplitude setting 09 and form setting 99.
4. Remove 200 µL from samples into a 1 x 250 µL v-bottom tray leaving blood spots behind.
5. Dry down samples over N_2.
6. Resuspend extracts in 100 µL of MeOH containing 10 mM ammonium formate.
7. Cover plate with alfoil and analyse samples by mass spectrometry.

EXAMPLE 3 MONITORING OF THERAPY FOR GAUCHER DISEASE USING SPHINGOLIPID AND PHOSPHOLIPID ANALYSIS

This report provides a detailed analysis of the initial trial of our developed methodology to monitor enzyme replacement therapy (ERT) in Gaucher disease using dried blood spots.

Patient samples: Dried blood spots were collected from Gaucher patients receiving ERT for up to 10 years. In addition, dried blood spots have been collected from patients not receiving ERT. Control samples were collected from healthy individuals. Total sample numbers are as shown in Table 10.

Sample preparation: From each Guthrie card sample 2x3 mm dried blood spots were punched and the lipids were eluted (16h) with 200 µL of isopropanol containing 200 nmol of each internal standard; Cer C17:0, GC(*d3*)C16:0, LC(*d3*)C16:0, PC C14:0, PG C14:0/14:0. The blood spots were removed and the isopropanol dried under a stream of nitrogen. Lipids were redissolved in 100 µL of methanol containing 10 mM NH_4COOH for analysis by mass spectrometry.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and

Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 μL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2 x 10^{-5} Torr. Lipids were analysed in +ve ion mode for sphingolipids and phosphatidylcholine and –ve ion mode for all other phospholipids. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species in addition to 36 phospholipid species were monitored using the ion pairs shown in Table 11 and 12. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Table 11 and 12).

RESULTS

To determine which analytes were potentially useful markers for monitoring Gaucher disease, the patients were grouped into control (group 1, n=22), Gaucher patients receiving ERT (group 2, n=68), and untreated Gaucher patients (group 3, n=20). Mann-Whitney U values were then calculated for each analyte to determine the difference between the control and untreated patients, control and treated patients, and treated and untreated patients. These results are shown in Table 13.

We observed that, in addition to the expected elevation of glucosylceramide (GC) in the untreated Gaucher patients compared to controls, there were significant differences in the level of ceramide C16:0, CTH C24:0 and the sphingomyelin species C16:0, C22:0 and C24:0 (all significant to the 0.01 level). With the exception of the ceramide C16:0, the same markers also showed a significant difference between treated and untreated Gaucher patients. Of the lactosylceramide species only the C16:0 and C22:0-OH species showed a significant difference between control and untreated patients (significant to the 0.05 level) (Table 13). While the GC and ceramide species were elevated in the Gaucher patient group compared to the control group, the LC, CTH and SM species showed a decrease in the Gaucher group. Many of the phospholipid species showed a significant difference between the control and Gaucher groups All of the

phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine species and many of the phosphatidylglycerol and phosphatidylinositol species were significantly decreased in the Gaucher patient group compared to the control group (Table 13). Many of these analytes were also decreased in the treated Gaucher patient group. For those analytes where a significant difference was observed between the control and Gaucher groups, the levels in the treated patients generally fell between the control and untreated patients. In each case ERT has partially normalised the lipid levels, although not in all patients.

Although the observed differences between control and untreated patients are significant there is still considerable overlap between the two populations. This is due, at least in part, to the range of lipid levels in the control and patient groups. To improve the discrimination of the markers we investigated the use of multiple markers by calculating Mann-Whitney U values for a number of ratios of different lipid species (Table 14).

In all ratios the Mann-Whitney U values were decreased compared to the GC C16:0 values or other single analytes (compare Table 14 with Table 13). Clearly, the use of multiple analytes or lipid profiles provides a better representation of lipid metabolism in control and Gaucher patients.

Discussion: In this study we have provided evidence that the primary storage substrate GC is a useful marker for monitoring Gaucher disease. We observe an increased level of GC in dried blood spots from untreated patients compared to controls and a normalisation of GC levels after ERT. This is an expected outcome, based on the known biochemistry of Gaucher disease. Somewhat less expected is the elevation in ceramide and the decrease in LC and sphingomyelin. We have previously reported that LC is decreased in the plasma of Gaucher patients and that the ratio of GC/LC provides a better discrimination of Gaucher patients from controls than the GC levels on their own (Whitfield et al 2002). In these preliminary studies we have identified that other lipids are also affected, these include not only ceramide and sphingomyelin but also a number of phospholipids. We have also shown that using a combination of these analytes with the GC and LC levels, provides greater discrimination and potentially a

better mechanism for monitoring ERT in Gaucher disease than the use of individual analytes.

Table 10. Patient and control samples included in this trial

Patient group	Number
Control	19
Treated Gaucher	68
Untreated Gaucher	20

Table 11. Lipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

Table 12. Phospholipid analytes used for Gaucher Monitoring

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
PC C32:0	PC C14:0	734.7/184
PC C32:1	PC C14:0	732.7/184
PC C34:1	PC C14:0	760.6/184
PC C34:2	PC C14:0	758.5/184
PC C36:2	PC C14:0	786.6/184
PC C36:4	PC C14:0	782.6/184
PC C38:4	PC C14:0	810.8/184
PC C14:0 (internal standard)		678.5/184
PE C18:0/20:4	PG C14:0/14:0	766.6/303.4
PE C18:1/18:1	PG C14:0/14:0	742.6/281.1
PG C16:0/18:1	PG C14:0/14:0	747.6/255.8
PG C16:0/22:6	PG C14:0/14:0	793.5/255.5
PG C16:1/18:1	PG C14:0/14:0	745.5/281.5
PG C16:1/20:4	PG C14:0/14:0	767.4/253.5
PG C18:1/18:0	PG C14:0/14:0	775.6/281.0
PG C18:1/18:1	PG C14:0/14:0	773.4/281.0
PG C18:1/18:2	PG C14:0/14:0	771.8/281.2
PG C18:1/20:4	PG C14:0/14:0	795.6/303.5
PG C18:1/22:5	PG C14:0/14:0	821.8/281.0
PG C18:1/22:6	PG C14:0/14:0	819.7/281.0
PG C18:2/22:6	PG C14:0/14:0	817.6/279.0
PG C20:4/22:6	PG C14:0/14:0	841.5/303.5
PG C22:6/22:5	PG C14:0/14:0	867.5/329.3
PG C22:6/22:6	PG C14:0/14:0	865.6/327.1
PI C16:0/18:0	PG C14:0/14:0	835.4/283.2
PI C16:0/20:4	PG C14:0/14:0	857.6/255.2
PI C18:0/18:0	PG C14:0/14:0	865.6/283.3
PI C18:0/18:1	PG C14:0/14:0	863.6/283.1
PI C18:0/20:4	PG C14:0/14:0	885.6/283.1
PI C18:0/22:4	PG C14:0/14:0	913.7/283.6
PI C18:0/22:5	PG C14:0/14:0	911.6/283.3
PI C18:1/18:1	PG C14:0/14:0	861.4/281.1
PI C18:1/20:4	PG C14:0/14:0	883.6/281.2
PS C16:0/16:0	PG C14:0/14:0	734.3/255.5
PS C18:0/20:4	PG C14:0/14:0	810.6/283.3
PS C18:1/18:0	PG C14:0/14:0	788.4/283.1
PG C14:0/14:0 (internal standard)		591.5/227.4

[a] PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine

Table 13. Mann-Whitney U values for lipid analytes between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

Analyte[d]	Control vs Gaucher		Control vs Gaucher treated		Gaucher vs Gaucher Treated	
	M-W U[e]	Sig.[f]	M-W U	Sig.	M-W U	Sig.
Cer C16:0	37	0.000	294	0.000	584	0.339
Cer C24:0	114	0.033	481	0.090	597	0.409
Cer C24:1	153	0.299	589	0.558	627	0.598
GC C16:0	25	0.000	310	0.001	332	0.001
GC C22:0	112	0.028	580	0.498	488	0.056
GC C24:0	125	0.068	606	0.681	493	0.063
GC C24:1	71	0.001	334	0.001	391	0.004
LC C16:0	120	0.049	556	0.355	534	0.146
LC C20:0	163	0.448	595	0.600	544	0.176
LC C22:0	178	0.736	589	0.558	667	0.897
LC C22:0-OH	111	0.026	578	0.485	494	0.064
LC C24:0	187	0.933	625	0.829	665	0.881
LC C24:1	166	0.500	596	0.607	670	0.921
(LC) CTH C16:0	124	0.064	594	0.593	508	0.087
(LC) CTH C18:0	174	0.653	563	0.394	622	0.564
(LC) CTH C20:0	139	0.152	440	0.034	611	0.492
(LC) CTH C22:0	115	0.035	573	0.453	486	0.053
(LC) CTH C24:0	76	0.001	462	0.059	418	0.009
(LC) CTH C24:1 (1134.9/264.4)	131	0.097	581	0.504	390	0.004
SM C16:0	69	0.001	497	0.126	379	0.003
SM C22:0	68	0.001	479	0.086	397	0.005
SM C24:0	85	0.003	353	0.003	464	0.031
PC C32:0	161	0.415	521	0.199	475	0.041
PC C32:1	47	0.000	236	0.000	678	0.984
PC C34:1	82	0.002	338	0.002	553	0.206
PC C34:2	70	0.001	432	0.028	437	0.016
PC C36:2	69	0.001	503	0.142	384	0.003
PC C36:4	48	0.000	322	0.001	401	0.005
PC C38:4	56	0.000	431	0.027	362	0.002
PE 18:0/20:4 (766.6/303.4)	57	0.000	509	0.025	325	0.000
PE 18:1/18:1 (742.6/281.1)	97	0.002	430	0.003	475.5	0.042
PG 16:0/18:1 (747.6/255.8)	160	0.131	715	0.757	538	0.157
PG 16:0/22:6 (793.5/255.5)	136.5	0.035	701	0.659	480	0.046
PG 16:1/18:1 (745.5/281.5)	97	0.002	386	0.001	541	0.166
PG 16:1/20:4 (767.4/253.5)	127	0.019	319	0.000	562	0.240
PG 18:1/18:0 (775.6/281.0)	133	0.028	604	0.176	539	0.160
PG 18:1/18:1 (773.4/281.0)	199	0.597	649	0.353	527	0.128
PG 18:1/18:2 (771.8/281.2)	104	0.003	488	0.015	520	0.111
PG 18:1/20:4 (795.6/303.5)	104	0.003	739	0.933	349	0.001
PG 18:1/22.:5 (821.8/281.0)	146	0.062	598	0.159	578	0.310

PG 18:1/22:6 (819.7/281.0)	140	0.044	540	0.051	600	0.426
PG 18:2/22:6 (817.6/279.0)	99	0.002	601	0.168	419	0.009
PG 20:4/22:6 (841.5/303.5)	82	0.001	692	0.599	316	0.000
PG 22:6/22:5 (867.5/329.3)	168	0.190	669.5	0.461	555	0.213
PG 22:6/22:6 (865.6/327.1)	174	0.247	491	0.016	605	0.455
PI 16:0/18:0 (835.4/283.2)	107	0.004	515	0.029	483	0.050
PI 16:0/20:4 (857.6/255.2)	96	0.002	532	0.043	501	0.075
PI 18:0/18:0 (865.6/283.3	125	0.017	463	0.007	617	0.530
PI 18:0/18:1 (863.6/283.1	69	0.000	359	0.000	607	0.467
PI 18:0/20:4 (885.6/283.1)	114	0.008	438	0.004	559	0.228
PI 18:0/22:4 (913.7/283.6)	166	0.174	488	0.015	671	0.929
PI 18:0/22:5 (911.6/283.3)	78	0.000	215	0.000	620	0.550
PI 18:1/18:1 (861.4/281.1)	99	0.002	499	0.019	522	0.116
PI 18:1/20:4 (883.6/281.2)	132	0.027	557	0.073	566	0.256
PS 16:0/16:0 (734.3/255.5)	188	0.420	589	0.135	605	0.455
PS 18:0/20:4 (810.6/283.3)	85	0.001	417	0.002	444	0.019
PS 18:1/18:0 (788.4/283.1)	81	0.000	409	0.001	556	0.217
Total Cer	150	0.261	597	0.615	632	0.633
Total GC	49	0.000	330	0.001	362	0.002
Total LC	170	0.574	619	0.781	630	0.619
Total CTH	103	0.015	519	0.192	475	0.041
Total SM	68	0.001	443	0.037	399	0.005
Total PC	75	0.001	397	0.011	445	0.019

[a] controls n=22
[b] untreated n= 20
[c] treated n= 68
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

Table 14. Mann-Whitney U values for lipid analyte ratios between controls[a], untreated Gaucher patients[b] and Gaucher patients treated with enzyme replacement therapy[c].

Analyte Ratio	Control vs Gaucher		Control vs Treated		Treated vs untreated	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
GC C16:0 / PE 18:0/20:4	28	0.000	241	0.000	291	0.000
GC C16:0 / PG 18:1/18:2	25	0.000	260	0.000	322	0.000
GC C16:0 / PG 20:4/20:6	19	0.000	344	0.002	229	0.000
GC C16:0 / PI 18:0/18:1	20	0.000	184	0.000	373	0.002
(Cer C16:0*GC C16:0) / (CTH C24:0*SM C16:0)	17	0.000	157	0.000	259	0.000
(Cer C16:0*GC C16:0) / (CTH C24:0*SM C16:0*PC32:1*PG20:4/22:6* PI18:0/18:1)	23	0.000	205	0.000	307	0.000
(Cer C16:0*GC C16:0) / (PC 32:1*PG 20:4/22:6*PI 18:0/18:1)	12	0.000	159	0.000	366	0.002

[a] controls n=22
[b] untreated n= 20
[c] treated n= 68
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

EXAMPLE 4

DIAGNOSIS OF FABRY DISEASE USING SPHINGOLIPID AND PHOSPHOLIPID ANALYSIS

This report summarises the results of analyses performed on urine, from controls, Fabry and Fabry heterozygotes, including analysis of phospholipids.

MATERIALS AND METHODS

Patient samples: Urine samples have been collected from 14 Fabry patients (two of whom have had renal transplants), 14 Fabry heterozygotes (three of whom had reported clinical symptoms) and 29 unaffected controls.

Sample preparation and analysis: Urine samples were prepared as described

To 1.5 mL urine add 5.6 mL $CHCl_3$/MeOH (1:2)

Add 400 pmol internal standards to each sample; 2 μL (d3) C16:0 LC (200 μM); 2 μL (d3) C16:0 GC (200 μM), 2 μL Cer C17:0 (200 μM), 2 μL PC (200 μM), 2 μL PG (200 μM) and 2 μL PI (200 μM).

Place tubes on platform shaker for 10 minutes at 150 opm. Stand tubes at room temperature for at least 50 minutes.

Partition with the addition of 1.9 mL $CHCl_3$ and 1.9 mL milliQ H_2O or KCl.

Place tubes on platform shaker for 10 minutes at 150 opm.

Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.

Wash the lower phase with the addition of 0.5 mL of Bligh-Dyer synthetic upper phase and vortexing briefly.

Centrifuge at 3000 rpm for 2 minutes then remove and discard upper phase by suction.

Dry samples (lower phase) under N_2 at 40°C (add water to heating block around tube to aid in evaporation). Periodically vortex the samples during the drying down process to ensure the highest recovery possible.

Resuspend extracts in 150 μL of MeOH containing 10 mM ammonium formate.

Mass spectrometry: Mass spectrometric analysis of lipids was performed using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 μL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2 x 10^{-5} Torr. Lipids were analysed in +ve ion mode for sphingolipids and phosphatidylcholine and –ve ion mode for all other phospholipids. Determination of lipids was performed using the multiple-reaction monitoring (MRM) mode. Seventeen different glycosphingolipid and ceramide species in addition to 36 phospholipid species were monitored using the ion pairs shown in Table 15 and 16. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Determination of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard

RESULTS

Analysis of Urine: Lipid profiling of the urine samples from control, Fabry and Fabry heterozygotes (Fabry het) has been performed. In all, 52 lipid species were determined including ceramide (Cer), glucosylceramide (GC), lactosylceramide (LC), trihexosylceramide (CTH), sphingomyelin (SM) and phosphatidylcholine (PC), phosphatidylglygerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS) species. Appropriate internal standards were used that provide quantification of these species (expressed as nmol/L urine). PC was included as a general marker of urinary sediment and all lipid species were subsequently corrected for total PC content and expressed as nmol/umol PC.

Table 17 shows the Mann-Whitney U values for each of the two patient groups compared to the control group and of the patient groups compared to each other. The data shows multiple analytes to be significantly different between the control and patient groups. Primarily LC CTH, PC and PG species show major differences between control and Fabry groups. Fewer species show significant differences between control and Fabry Het groups but still 11 lipid species show a significance less than 0.01.

Table 18 shows the Mann-Whitney U values for different lipid ratios involving 2 or more lipid species. In most instances the ratios provide better discrimination than the individual analytes involved (based on the Mann-Whitney U values.

DISCUSSION

In this study we have provided evidence that the primary storage substrate CTH is a useful marker for diagnosis of Fabry disease. We observe an increased level of CTH in urine from most Fabry patients. This is an expected outcome, based on the known biochemistry of Fabry disease. Somewhat less expected is the elevation in all of the PC and PG species as well as two ceramide species and two of the three sphingomyelin species. In these preliminary studies we have identified that in addition to CTH, other lipids are also affected, these include not only ceramide and sphingomyelin but also a number of phospholipids. We have also shown that using a combination of these analytes either alone or with the CTH levels, provides greater discrimination and potentially a better mechanism for diagnosis of Fabry and identification of Fabry heterozygotes than the use of individual analytes.

Table 15. Lipid analytes used for Fabry urine analysis

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		552.7/264.4
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		703.8/264.4
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
LC(*d3*)C16:0 (internal standard)		865.6/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C14:0 (internal standard)		678.5/184.1

[a] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine

Table 16. Phospholipid analytes used for Fabry urine analysis.

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
PC C32:0	PC C14:0	734.7/184
PC C32:1	PC C14:0	732.7/184
PC C34:1	PC C14:0	760.6/184
PC C34:2	PC C14:0	758.5/184
PC C36:2	PC C14:0	786.6/184
PC C36:4	PC C14:0	782.6/184
PC C38:4	PC C14:0	810.8/184
PC C14:0 (internal standard)		678.5/184.1
PE C18:0/20:4	PG C14:0/14:0	766.6/303.4
PE C18:1/18:1	PG C14:0/14:0	742.6/281.1
PG C16:0/18:1	PG C14:0/14:0	747.6/255.8
PG C16:0/22:6	PG C14:0/14:0	793.5/255.5
PG C16:1/18:1	PG C14:0/14:0	745.5/281.5
PG C16:1/20:4	PG C14:0/14:0	767.4/253.5
PG C18:1/18:0	PG C14:0/14:0	775.6/281.0
PG C18:1/18:1	PG C14:0/14:0	773.4/281.0
PG C18:1/18:2	PG C14:0/14:0	771.8/281.2
PG C18:1/20:4	PG C14:0/14:0	795.6/303.5
PG C18:1/22:5	PG C14:0/14:0	821.8/281.0
PG C18:1/22:6	PG C14:0/14:0	819.7/281.0
PG C18:2/22:6	PG C14:0/14:0	817.6/279.0
PG C20:4/22:6	PG C14:0/14:0	841.5/303.5
PG C22:6/22:5	PG C14:0/14:0	867.5/329.3
PG C22:6/22:6	PG C14:0/14:0	865.6/327.1
PG C14:0/14:0 (internal standard)		591.5/227.4
PI C16:0/18:0	PI C16:0/16:0	835.4/283.2
PI C16:0/20:4	PI C16:0/16:0	857.6/255.2
PI C18:0/18:0	PI C16:0/16:0	865.6/283.3
PI C18:0/18:1	PI C16:0/16:0	863.6/283.1
PI C18:0/20:4	PI C16:0/16:0	885.6/283.1
PI C18:0/22:4	PI C16:0/16:0	913.7/283.6
PI C18:0/22:5	PI C16:0/16:0	911.6/283.3
PI C18:1/18:1	PI C16:0/16:0	861.4/281.1
PI C18:1/20:4	PI C16:0/16:0	883.6/281.2
PI C14:0/14:0 (internal standard)		751.5/227.4
PS C16:0/16:0	PG C14:0/14:0	734.3/255.5
PS C18:0/20:4	PG C14:0/14:0	810.6/283.3
PS C18:1/18:0	PG C14:0/14:0	788.4/283.1

[a] PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine

Table 17. Mann-Whitney U values for lipid analytes between controls[a], Fabry[b] and Fabry Hets[c].

Analyte[d]	Cont vs Fabry		Cont vs Het		Fabry vs Het	
	M-W U[e]	Sig[f]	M-W U	Sig	M-W U	Sig
Cer C16:0 (538.7/264.4)	119	0.029	189	0.717	62	0.098
Cer C24:0 (650.7/264.4)	132	0.066	175	0.468	52	0.035
Cer C24:1 (648.7/264.4)	70	0.001	187	0.678	37	0.005
Cer C20:0 (592.7/264.4)	155	0.213	168	0.364	59	0.073
Cer C20:1 (590.7/264.4)	193	0.795	124	0.041	46	0.017
Cer C23:0 (636.7/264.4)	144	0.126	143	0.120	53	0.039
Cer C23:1 (634.8/264.4)	160	0.265	146	0.140	51	0.031
GC C16:0 (700.6/264.4)	203	1.000	148	0.154	70	0.198
GC C22:0 (784.7/264.4)	152	0.186	89	0.003	48	0.022
GC C24:0 (812.7/264.4)	182	0.586	101	0.008	60	0.081
GC C24:1 (810.8/264.4)	137	0.087	143	0.120	41	0.009
LC C16:0 (862.4/264.4)	107	0.013	117	0.026	93	0.818
LC C20:0 (918.7/264.4)	66	0.000	196	0.856	29	0.002
LC C22:0 (946.7/264.4)	70	0.001	151	0.178	53	0.039
LC C22:0-OH (962.7/264.4)	75	0.001	166	0.338	44	0.013
LC C24:0 (974.8/264.4)	11	0.000	100	0.008	19	0.000
LC C24:1 (972.8/264.4)	41	0.000	98	0.007	66	0.141
(LC) CTH C16:0 (1024.8/264.4)	41	0.000	143	0.120	39	0.007
(LC) CTH C18:0 (1052.7/264.4)	18	0.000	157	0.233	20	0.000
(LC) CTH C20:0 (1080.9/264.4)	75	0.001	197	0.876	32	0.002
(LC) CTH C22:0 (1108.9/264.4)	47	0.000	96	0.006	48	0.022
(LC) CTH C24:0 (1136.9/264.4)	26	0.000	111	0.017	34	0.003
(LC) CTH C24:1 (1134.9/264.4)	43	0.000	106	0.012	46	0.017
PC C32:0 (734.7/184.1)	118	0.028	166	0.338	77	0.335
PC C32:1 (732.7/184.1)	58	0.000	167	0.351	55	0.048
PC C34:1 (760.6/184.1)	83	0.002	113	0.020	87	0.613
PC C34:2 (758.5/184.1)	86	0.002	183	0.604	34	0.003
PC C36:2 (786.6/184.1)	125	0.043	130	0.058	82	0.462
PC C36:4 (782.6/184.1)	87	0.003	202	0.979	59	0.073
PC C38:4 (810.8/184.1)	65	0.000	199	0.917	49	0.024
SM C16:0 (703.9/184.1)	182	0.586	160	0.265	84	0.520
SM C22:0 (787.8/184.1)	58	0.000	126	0.046	94	0.854
SM C24:0 (815.8/184.1)	44	0.000	100	0.008	97	0.963
PG C16:0/18:1 (747.6/255.8)	75	0.001	115	0.023	61	0.089
PG C16:0/22:6 (793.5/255.5)	70	0.001	154	0.204	54	0.043
PG C16:1/18:1 (745.5/281.5)	90	0.003	82	0.002	67	0.154
PG C16:1/20:4 (767.4/253.5)	137	0.087	193	0.795	70	0.198
PG C18:1/18:0 (775.6/281.0)	28	0.000	73	0.001	51	0.031
PG C18:1/18:1 (773.4/281.0)	15	0.000	73	0.001	38	0.006
PG C18:1/18:2 (771.8/281.2)	15	0.000	69	0.001	42	0.010
PG C18:1/20:4 (795.6/303.5)	31	0.000	126	0.046	48	0.022
PG C18:1/22.:5 (821.8/281.0)	20	0.000	109	0.015	38	0.006

PG C18:1/22:6 (819.7/281.0)	21	0.000	138	0.092	22	0.000
PG C18:2/22:6 (817.6/279.0)	25	0.000	155	0.213	23	0.001
PG C20:4/22:6 (841.5/303.5)	30	0.000	186	0.659	26	0.001
PG C22:5/22:5 (869.6/329.3)	9	0.000	190	0.736	9	0.000
PG C22:6/22:5 (867.5/329.3)	20	0.000	200	0.938	11	0.000
PG C22:6/22:6 (865.6/327.1)	30	0.000	193	0.795	23	0.001
PI C16:0/18:0 (835.4/283.2)	147	0.147	174	0.452	73	0.251
PI C16:0/20:4 (857.6/255.2)	191	0.756	138	0.092	60	0.081
PI C18:0/18:0 (865.6/283.3)	49	0.000	139	0.097	14	0.000
PI C18:0/18:1 (863.6/283.1)	197	0.876	170	0.392	79	0.383
PI C18:0/20:3 (887.6/283.1)	185	0.641	137	0.087	65	0.129
PI C18:0/20:4 (885.6/283.1)	193	0.795	123	0.038	54	0.043
PI C18:0/22:5 (911.6/283.3)	167	0.351	144	0.126	55	0.048
PI C18:1/18:1 (861.4/281.1)	153	0.195	188	0.697	74	0.270
PI C18:1/20:4 (883.6/281.2)	201	0.959	149	0.162	68	0.168
PS C16:0/16:0 (734.3/255.5)	131	0.062	175	0.468	63	0.108
PS C18:1/18:0 (788.4/283.1)	57	0.000	103	0.010	69	0.183
PE C18:0/20:4 (766.6/303.4)	153	0.195	154	0.204	96	0.927
PE C18:1/18:1 (742.6/281.1)	151	0.178	199	0.917	70	0.198
total Cer	117	0.026	199	0.917	60	0.081
TOTAL_GC	197	0.876	103	0.010	52	0.035
TOTAL_LC	36	0.000	123	0.038	42	0.010
total CTH	43	0.000	130	0.058	40	0.008
TOTALPC	203	1.000	203	1.000	98	1.000
TOTAL_SM	72	0.001	129	0.055	97	0.963
TOTAL_PG	15	0.000	97	0.006	35	0.004
TOTAL_PI	127	0.049	137	0.087	41	0.009
TOTAL_PE	146	0.140	187	0.678	75	0.291
TOTAL_PS	59	0.000	106	0.012	70	0.198

[a] controls n=29
[b] Fabrt n= 14
[c] Fabry Het n= 14
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

Table 18. Mann-Whitney U values for lipid analyte ratios between controls[a], Fabry[b] and Fabry Hets[c].

Analyte[d]	Control v Fabry		Control v Fabry Het		Fabry v Fabry Het	
	M-W U[e]	Sig.[f]	M-W U	Sig.	M-W U	Sig.
CTH C24:1/SM C24:0	18	0.000	51	0.000	39	0.007
LC C24:1/GC C24:0	16	0.000	65	0.000	81	0.435
PC C38:4/PC C32:1	58	0.000	187	0.678	55	0.048
PC C36:4*PC C38:4/PC C32:1*PC C34:1	56	0.000	182	0.586	55	0.048
CTH C24:1/SM C24:0/LC C24:1/ GC C24:0	83	0.002	191	0.756	42	0.010
PG C18:1/18:1 /PS C18:1/18:0	2	0.000	35	0.000	14	0.000
PI C18:0/18:0 / PS C18:1/18:0	10	0.000	195	0.836	8	0.000
PG C18:1/18:1* PI C18:0/18:0 / PS C18:1/18:0	1	0.000	106	0.012	8	0.000
PG C18:1/18:1 / SM C18:1/18:0	4	0.000	16	0.000	33	0.003

[a] controls n=29
[b] Fabrt n= 14
[c] Fabry Het n= 14
[d] Cer = ceramide, GC = glucosylceramide, LC = lactosylceramide, CTH = ceramide trihoxoside, SM = sphingomyelin, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine, PE phosphatidylethanolamine
[e] Mann-Whitney U values
[f] significance (two-tailed)

EXAMPLE 5

PATIENT EVALUATION AND MONITORING OF THERAPY FOR FABRY DISEASE

This example provides results of studies to examine the effect of therapy on the lipid profile in plasma and urine from Fabry hemizygotes and heterozygotes.

MATERIALS AND METHODS

Plasma samples were collected from:

- Control adults (19) taken from members of the Department of Genetic Medicine, Children, Youth and Women's Health Service (CYWHS), Adelaide, and control samples (19) taken from patients referred to the Department for diagnosis but were subsequently shown not to have a lysosomal storage disorder;
- Fabry hemizygotes (25) and known heterozygotes (3) within Australia;
- Fabry hemizygotes (5) and heterozygotes (10) who are receiving therapy in Germany.

Urine samples were collected from:

- Control adults and children (28) taken from members of the Department of Genetic Medicine, CYWHS, Adelaide, and their families.
- Fabry hemizygotes (13) and known heterozygotes (19) within Australia;
- Fabry hemizygotes (5) and heterozygotes (10) who are receiving therapy in Germany;

Sample preparation: Lipids were extracted from plasma (100 μL) using the method of Folch and from urine (1.5 mL) using the method of Bligh/Dyer.

Mass spectrometry: A range of lipids were analysed by mass spectrometry (Tables 19 and 20) using a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source and Analyst data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of methanol at a flow rate of 80 μL/minute. For all analytes nitrogen was used as the collision gas at a pressure 2×10^{-5} Torr. Lipids were analysed in +ve ion mode (Cer, GC, LC, CTH, SM, PC) or –ve ion mode (gangliosides, PG, PI, PE, PS). Lipid analysis was performed using the multiple-reaction monitoring (MRM) mode. Lipid species were monitored using the ion pairs shown in Tables 2 and 3. Each ion pair was monitored for 100 milliseconds and the measurements were repeated and averaged over the injection period. Measurement of lipids was achieved by relating the peak height of each lipid ion signal to the peak height of the signal from the corresponding internal standard (Tables 19 and 20).

RESULTS

Table 21 shows the mean plasma concentrations of each analyte from control and Fabry hemizygotes, Fabry heterozygotes, hemizygotes on ERT and heterozygotes on ERT. Also included is the ratio of the hemizygote value over the control value, and the heterozygote value over the control value. These ratios indicate which analytes are increased in the disease state and which are decreased. Clearly, the CTH species show an increase in the hemizygote and heterozygote populations compared to the control

group and this change is determined to be significant for all species in the hemizygotes by the Mann-Whitney U values shown in Table 22. Interestingly, the Mann-Witney U values for the control versus the treated hemizygotes and heterozygotes indicate that the CTH levels in the treated patients are not completely normalised. This is also evident in Figure 23.

In addition to CTH, a number of PG species were also elevated, particularly in the heterozygotes (Table 21); these were also statistically significant based on the Mann-Whitney U values (Table 22). A number of analytes were also decreased in the hemizygote, and to a lesser extent in the heterozygote groups, compared to the control group. These include some PC species, G_{M3} species, as well as PI, PE and PS species (Table 21). However, most analytes showed considerable over-lap between the control and affected groups (Figure 24).

The ability of a number of lipid ratios to distinguish between control and affected groups was also examined (Table 22) and these generally provided better discrimination than the individual lipid species. A number of lipid ratios were plotted against each other to establish whether or not there was correction in the ERT-treated patients (Figure 25). In each plot a clear trend toward the normal lipid profile was observed for the hemizygous patients on ERT; heterozygotes were closer to normal without ERT and showed no significant change with ERT.

A similar analysis was performed on the lipid profiles observed in urine from the control and patient groups. The lipid analytes were normalised to the total level of PC to compensate for the differing levels of urinary sediment in each sample. In addition to the CTH species, significant elevations were observed in a number of other lipid types including some Cer species, LC and a number of PG species. Simultaneously, a significant decrease was observed in the level of PS 18:1/18:0 in both the hemizygote and heterozygote groups compared to the control group (Tables 23 and 24). The plasma data revealed relatively little change in CTH levels following ERT; the urine data reflected a similar pattern between treated and untreated patient groups (Figure 26a). This trend was also borne out for most other lipid analytes (Figure 26 and Table 24).

Plotting one analyte against another (Figure 27a) or plotting ratios of analytes (Figures 27b and c) improved discrimination between control and affected patient groups. In particular, the multiple ratios shown in Figure 27c most clearly discriminated between the control and affected groups, thus demonstrating the potential of the phospholipid species in improving discrimination between Fabry hemizygotes and heterozygotes from controls.

DISCUSSION

Our studies on Fabry disease have demonstrated that the lipid profile in plasma and urine is significantly altered in both hemizygotes and heterozygotes. We have also shown that the altered urinary lipid profile can be used to identify heterozygotes from the control population and that the plasma lipid profile in Fabry hemizygotes is partially normalised upon enzyme replacement therapy. Thus Lipid profiling has application in the monitoring the efficacy of therapy in Fabry disease.

Table 19. Lipid analytes used for analysis of Fabry samples.

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
Cer C16:0	Cer C17:0	538.7/264.4
Cer C23:0	Cer C17:0	636.7/264.4
Cer C23:1	Cer C17:0	634.7/264.4
Cer C24:0	Cer C17:0	650.7/264.4
Cer C24:1	Cer C17:0	648.7/264.4
Cer C17:0 (internal standard)		**552.7/264.4**
GC C16:0	GC(*d3*)C16:0	700.6/264.4
GC C22:0	GC(*d3*)C16:0	784.7/264.4
GC C24:0	GC(*d3*)C16:0	812.7/264.4
GC C24:1	GC(*d3*)C16:0	810.8/264.4
GC(*d3*)C16:0 (internal standard)		**703.8/264.4**
LC C16:0	LC(*d3*)C16:0	862.4/264.4
LC C20:0	LC(*d3*)C16:0	918.6/264.4
LCC22:0	LC(*d3*)C16:0	946.7/264.4
LC C22:0-OH	LC(*d3*)C16:0	962.7/264.4
LC C24:0	LC(*d3*)C16:0	974.8/264.4
LC C24:1	LC(*d3*)C16:0	972.8/264.4
LC(*d3*)C16:0 (internal standard)		**865.6/264.4**
CTH C16:0	LC(*d3*)C16:0	1024.1/264.4
CTH C18:0	LC(*d3*)C16:0	1052.1/264.4
CTH C20:0	LC(*d3*)C16:0	1080.1/264.4
CTH C22:0	LC(*d3*)C16:0	1108.1/264.4
CTH C24:0	LC(*d3*)C16:0	1136.6/264.4
CTH C24:1	LC(*d3*)C16:0	1134.1/264.4
SM C16:0	PC C14:0	703.9/184.1
SM C22:0	PC C14:0	787.8/184.1
SM C24:0	PC C14:0	815.8/184.1
PC C32:0	PC C14:0	706.5/184.1
PC C32:1	PC C14:0	704.5/184.1
PC C34:1	PC C14:0	732.5/184.1
PC C34:2	PC C14:0	730.5/184.1
PC 36:2	PC C14:0	758.6/184.1
PC C36:4	PC C14:0	754.6/184.1
PC C38:4	PC C14:0	782.6/184.1
PC C14:0[b] (internal standard)		**678.5/184.1**

[a] Cer = ceramide; GC = glucosylceramide; LC = lactosylceramide; CTH = ceramide trihexoside; SM = sphingomyelin; PC = phosphatidylcholine

[b] PC C14:0 is a commercial standard and is known to have a C16:0 second fatty acid (equivalent to PC C30:0)

Table 20. Lipid analytes used for analysis of Fabry samples

Lipid analytes[a]	Internal standard	MRM ion pairs (*m/z*)
GM3 C16:0	GM2 C22:1	1151.9/290.0
GM3 C22:0	GM2 C22:1	1235.9/290.0
GM3 C24:0	GM2 C22:1	1263.1/290.0
GM3 C24:1	GM2 C22:1	1261.6/290.0
GM2 C22:1 (Internal Standard)		**1383.0/290.0**
PG 16:0/18:1	PG 14:0/14:0	747.6/255.8
PG 16:0/22:6	PG 14:0/14:0	793.5/255.5
PG 16:1/18:1	PG 14:0/14:0	745.5/281.5
PG 16:1/20:4	PG 14:0/14:0	767.4/253.5
PG 18:1/18:0	PG 14:0/14:0	775.6/281.0
PG 18:1/18:1	PG 14:0/14:0	773.4/281.0
PG 18:1/18:2	PG 14:0/14:0	771.8/281.2
PG 18:1/20:4	PG 14:0/14:0	795.6/303.5
PG 18:1/22.:5	PG 14:0/14:0	821.8/281.0
PG 18:1/22:6	PG 14:0/14:0	819.7/281.0
PG 18:2/22:6	PG 14:0/14:0	817.6/279.0
PG 20:4/22:6	PG 14:0/14:0	841.5/303.5
PG 22:5/22:5	PG 14:0/14:0	869.6/329.3
PG 22:6/22:5	PG 14:0/14:0	867.5/329.3
PG 22:6/22:6	PG 14:0/14:0	865.6/327.1
PG 14:0/14:0 (Internal Standard)		**665.2/227**
PI 16:0/18:0	PI 16:0/16:0	835.4/283.2
PI 16:0/20:4	PI 16:0/16:0	857.6/255.2
PI 18:0/18:0	PI 16:0/16:0	865.6/283.3
PI 18:0/18:1	PI 16:0/16:0	863.6/283.1
PI 18:0/20:3	PI 16:0/16:0	887.6/283.1
PI 18:0/20:4	PI 16:0/16:0	885.6/283.1
PI 18:0/22:5	PI 16:0/16:0	911.6/283.3
PI 18:1/18:1	PI 16:0/16:0	861.4/281.1
PI 18:1/20:4	PI 16:0/16:0	883.6/281.2
PS 16:0/16:0	PI 16:0/16:0	734.3/255.5
PS 18:1/18:0	PI 16:0/16:0	788.4/283.1
PE 18:0/20:4	PI 16:0/16:0	766.6/303.4
PE 18:1/18:1	PI 16:0/16:0	742.6/281.1
PI 16:0/16:0 (Internal Standard)		**809.5/255.1**

[a] GM3 = G_{M3} ganglioside; GM2 = G_{M2} ganglioside; PG = phosphatidylglycerol/lysobisphosphatidic acid; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine.

Table 21. Mean lipid concentrations[a] present in plasma from control and Fabry patients.

Analyte	Control (n= 38) (nM)	Hemi (n=25) (nM)	Het (n=3) (nM)	Hemi (ERT) (N=5) (nM)	Het (ERT) (N=10) (nM)	Hemi/ Cont	Het/ Cont
Cer C16:0 (538.7/264.4)	279	159	291	223	254	0.6	1.0
Cer C20:0 (592.7/264.4)	6	4	5	4	5	0.7	0.8
Cer C20:1 (590.7/264.4)	8	7	8	8	8	0.8	0.9
Cer C23:0 (636.7/264.4)	866	611	1046	688	844	0.7	1.2
Cer C23:1 (634.8/264.4)	55	38	62	42	47	0.7	1.1
Cer C24:0 (650.7/264.4)	3069	1880	3908	2272	2855	0.6	1.3
Cer C24:1 (648.7/264.4)	1204	670	1199	1123	1326	0.6	1.0
GC C16:0 (700.6/264.4)	793	714	941	857	1125	0.9	1.2
GC C18:0 (728.6/264.4)	123	126	145	145	180	1.0	1.2
GC C20:0 (756.8/264.4)	90	95	108	130	133	1.1	1.2
GC C22:0 (784.7/264.4)	764	887	1085	1187	1263	1.2	1.4
GC C24:0 (812.7/264.4)	1056	1156	1257	1544	1644	1.1	1.2
GC C24:1 (810.8/264.4)	833	783	762	1099	1181	0.9	0.9
LC C16:0 (862.4/264.4)	24326	15998	23618	21208	25249	0.7	1.0
LC C20:0 (918.7/264.4)	613	542	683	631	631	0.9	1.1
LC C22:0 (946.7/264.4)	2172	1365	1632	1617	1765	0.6	0.8
LC C22:0-OH (962.7/264.4)	329	305	366	294	373	0.9	1.1
LC C24:0 (974.8/264.4)	2552	1752	2200	2138	2112	0.7	0.9
LC C24:1 (972.8/264.4)	5443	3984	4571	5422	5063	0.7	0.8
(LC) CTH C16:0 (1024.8/264.4)	3752	9979	4906	6571	5300	2.7	1.3
(LC) CTH C18:0 (1052.7/264.4)	717	2000	1118	1543	962	2.8	1.6
(LC) CTH C20:0 (1080.9/264.4)	278	635	433	542	368	2.3	1.6
(LC) CTH C22:0 (1108.9/264.4)	827	2275	1174	1431	1073	2.7	1.4
(LC) CTH C24:0 (1136.9/264.4)	1031	3086	1346	2153	1339	3.0	1.3
(LC) CTH C24:1 (1134.9/264.4)	1474	2868	1684	2795	2146	1.9	1.1
PC C32:0 (734.7/184.1)	14260	8170	12840	11386	13407	0.6	0.9
PC C32:1 (732.7/184.1)	21384	12028	21104	19133	22886	0.6	1.0
PC C34:1 (760.6/184.1)	217075	128374	206524	185194	222028	0.6	1.0
PC C34:2 (758.5/184.1)	293189	150908	250741	240057	304193	0.5	0.9
PC C36:2 (786.6/184.1)	200390	101723	175606	159491	197896	0.5	0.9
PC C36:4 (782.6/184.1)	136221	27803	108696	100642	142719	0.2	0.8
PC C38:4 (810.8/184.1)	51176	12147	44030	41911	55820	0.2	0.9
SM C16:0 (703.9/184.1)	26669	20906	34769	25435	28859	0.8	1.3
SM C22:0 (787.8/184.1)	84184	44487	83880	64927	79815	0.5	1.0
SM C24:0 (815.8/184.1)	17724	11448	21421	15114	17546	0.6	1.2
GM3 C16:0 (1151.9/290.0)	9652	6771	10765	7858	8398	0.7	1.1
GM3 C22:0 (1235.9/290.0)	11	9	12	6	4	0.8	1.1
GM3 C24:0 (1263.1/290.0)	3216	1269	2318	1183	1237	0.4	0.7
GM3 C24:1 (1261.6/290.0)	4308	1846	3280	1620	1634	0.4	0.8

Table 21 cont....

Analyte	Control (N=38) (nM)	Hemi (N=25) (nM)	Het (N=3) (nM)	Hemi (ERT) (N=5) (nM)	Het (ERT) (N=10) (nM)	Hemi/ Cont	Het/ Cont
PG 16:0/18:1 (747.6/255.8)	2	2	2	2	2	1.5	1.3
PG 16:0/22:6 (793.5/255.5)	75	59	95	61	76	0.8	1.3
PG 16:1/18:1 (745.5/281.5)	47	29	59	38	60	0.6	1.3
PG 16:1/20:4 (767.4/253.5)	5	4	5	5	7	0.8	0.9
PG 18:1/18:0 (775.6/281.0)	46	42	76	42	56	0.9	1.7
PG 18:1/18:1 (773.4/281.0)	47	49	57	40	51	1.1	1.2
PG 18:1/18:2 (771.8/281.2)	21	19	28	16	19	0.9	1.4
PG 18:1/20:4 (795.6/303.5)	13	7	15	8	11	0.6	1.2
PG 18:1/22.:5 (821.8/281.0)	33	34	57	30	32	1.1	1.7
PG 18:1/22:6 (819.7/281.0)	45	53	107	68	60	1.2	2.3
PG 18:2/22:6 (817.6/279.0)	42	42	71	58	55	1.0	1.7
PG 20:4/22:6 (841.5/303.5)	7	6	14	12	11	0.9	1.9
PG 22:5/22:5 (869.6/329.3)	2	1	1	1	1	0.4	0.9
PG 22:6/22:5 (867.5/329.3)	1	1	2	2	1	0.7	1.5
PG 22:6/22:6 (865.6/327.1)	2	2	4	3	2	0.7	1.7
PI 16:0/18:0 (835.4/283.2)	1273	1912	2685	2636	1784	1.5	2.1
PI 16:0/20:4 (857.6/255.2)	1314	280	1175	1146	1212	0.2	0.9
PI 18:0/18:0 (865.6/283.3)	62	73	107	80	71	1.2	1.7
PI 18:0/18:1 (863.6/283.1)	1558	843	1775	1348	1321	0.5	1.1
PI 18:0/20:3 (887.6/283.1)	2753	791	2514	2056	2534	0.3	0.9
PI 18:0/20:4 (885.6/283.1)	13578	2908	11159	10768	12644	0.2	0.8
PI 18:0/22:5 (911.6/283.3)	428	98	336	343	337	0.2	0.8
PI 18:1/18:1 (861.4/281.1)	1307	683	1124	1038	991	0.5	0.9
PI 18:1/20:4 (883.6/281.2)	831	166	573	472	480	0.2	0.7
PS 16:0/16:0 (734.3/255.5)	2	11	12	4	3	5.6	6.2
PS 18:1/18:0 (788.4/283.1)	167	10	19	9	10	0.1	0.1
PE 18:0/20:4 (766.6/303.4)	279	36	204	202	420	0.1	0.7
PE 18:1/18:1 (742.6/281.1)	220	64	181	111	238	0.3	0.8
Total Cer	5487	3370	6519	4361	5339	0.6	1.2
Total GC	3658	3760	4299	4963	5526	1.0	1.2
Total LC	35435	23946	33070	31309	35192	0.7	0.9
Total PC	933695	441153	819541	757814	958949	0.5	0.9
Total CTH	8080	20844	10661	15035	11188	2.6	1.3
Total GM3	17188	9894	16375	10667	11273	0.6	1.0
Total PG	388	351	593	385	446	0.9	1.5
Total PI	23104	7755	21449	19889	21372	0.3	0.9
Total PS	169	21	31	13	13	0.1	0.2
Total PE	498	100	385	314	658	0.2	0.8

[a] Determination of lipid species was semi-quantitative (see Results and Discussion).

Table 22. Statistical analysis of lipid levels in plasma samples from control, Fabry hemizygotes, Fabry heterozygotes, Fabry hemizygotes on ERT and Fabry heterozygotes on ERT.
Control (n=38); Hemi (n=25); Het (n=3); Hemi (ERT) (n=5); Het (ERT) (N=10)

Analyte / Ratio	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
Cer C16:0 (538.7/264.4)	71	0.000	42	0.453	50	0.088	116	0.060	21	0.021	7	0.176
Cer C20:0 (592.7/264.4)	293	0.011	43	0.483	60	0.185	131	0.134	54	0.636	12	0.612
Cer C20:1 (590.7/264.4)	354	0.089	56	0.960	91	0.880	163	0.493	43	0.278	7	0.176
Cer C23:0 (636.7/264.4)	177	0.000	26	0.121	63	0.225	147	0.275	40	0.211	6	0.128
Cer C23:1 (634.8/264.4)	170	0.000	38	0.342	52	0.103	98	0.020	39	0.191	6	0.128
Cer C24:0 (650.7/264.4)	134	0.000	24	0.099	46	0.063	143	0.233	31	0.080	4	0.063
Cer C24:1 (648.7/264.4)	120	0.000	55	0.920	86	0.733	148	0.286	15	0.008	14	0.866
GC C16:0 (700.6/264.4)	322	0.032	24	0.099	60	0.185	36	0.000	31	0.080	6	0.128
GC C18:0 (728.6/264.4)	475	1.000	31	0.193	60	0.185	50	0.000	40	0.211	7	0.176
GC C20:0 (756.8/264.4)	468	0.922	21	0.072	22	0.006	36	0.000	25	0.037	2	0.028
GC C22:0 (784.7/264.4)	371	0.144	11	0.021	20	0.004	23	0.000	26	0.042	8	0.237
GC C24:0 (812.7/264.4)	408	0.347	34	0.250	18	0.004	27	0.000	21	0.021	5	0.091
GC C24:1 (810.8/264.4)	396	0.267	48	0.652	34	0.021	68	0.002	24	0.032	4	0.063
LC C16:0 (862.4/264.4)	151	0.000	55	0.920	58	0.161	183	0.859	25	0.037	14	0.866
LC C20:0 (918.7/264.4)	397	0.273	36	0.293	75	0.449	155	0.374	35	0.126	10	0.398
LC C22:0 (946.7/264.4)	210	0.000	44	0.515	74	0.426	184	0.879	21	0.021	12	0.612
LC C22:0-OH (962.7/264.4)	471	0.950	37	0.317	89	0.820	146	0.264	56	0.718	14	0.866
LC C24:0 (974.8/264.4)	189	0.000	48	0.652	74	0.426	143	0.233	28	0.055	12	0.612
LC C24:1 (972.8/264.4)	213	0.000	42	0.453	88	0.791	158	0.417	27	0.048	14	0.866
(LC) CTH C16:0 (1024.8/264.4)	148	0.000	32	0.211	19	0.004	62	0.001	45	0.330	15	1.000
(LC) CTH C18:0 (1052.7/264.4)	103	0.000	15	0.035	12	0.002	81	0.006	55	0.676	11	0.499
(LC) CTH C20:0 (1080.9/264.4)	129	0.000	0	0.004	25	0.008	65	0.002	60	0.889	3	0.043
(LC) CTH C22:0 (1108.9/264.4)	98	0.000	13	0.028	39	0.034	105	0.031	39	0.191	9	0.310
(LC) CTH C24:0 (1136.9/264.4)	77	0.000	22	0.080	33	0.019	133	0.148	48	0.420	11	0.499
(LC) CTH C24:1 (1134.9/264.4)	170	0.000	36	0.293	20	0.004	81	0.006	53	0.597	9	0.310

Table 22 cont....

Analyte / Ratio	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
PC C32:0 (734.7/184.1)	101	0.000	43	0.483	56	0.140	172	0.648	28	0.055	13	0.735
PC C32:1 (732.7/184.1)	117	0.000	53	0.841	72	0.384	172	0.648	33	0.101	12	0.612
PC C34:1 (760.6/184.1)	95	0.000	51	0.764	65	0.256	179	0.780	25	0.037	12	0.612
PC C34:2 (758.5/184.1)	64	0.000	41	0.423	40	0.037	185	0.899	16	0.010	10	0.398
PC C36:2 (786.6/184.1)	55	0.000	40	0.395	37	0.028	171	0.630	18	0.013	15	1.000
PC C36:4 (782.6/184.1)	6	0.000	46	0.582	39	0.034	177	0.741	2	0.001	11	0.499
PC C38:4 (810.8/184.1)	5	0.000	52	0.802	50	0.088	157	0.402	2	0.001	12	0.612
SM C16:0 (703.9/184.1)	202	0.000	6	0.011	91	0.880	151	0.322	36	0.140	4	0.063
SM C22:0 (787.8/184.1)	64	0.000	52	0.802	37	0.028	149	0.298	21	0.021	14	0.866
SM C24:0 (815.8/184.1)	91	0.000	31	0.193	67	0.289	172	0.648	29	0.062	9	0.310
GM3 C16:0 (1151.9/290.0)	257	0.002	37	0.317	72	0.384	167	0.559	44	0.303	6	0.128
GM3 C22:0 (1235.9/290.0)	353	0.087	54	0.881	43	0.049	27	0.000	39	0.191	4	0.063
GM3 C24:0 (1263.1/290.0)	34	0.000	34	0.250	2	0.000	10	0.000	58	0.802	6	0.128
GM3 C24:1 (1261.6/290.0)	57	0.000	36	0.293	2	0.000	10	0.000	55	0.676	7	0.176
PG 16:0/18:1 (747.6/255.8)	410	0.361	24	0.099	88	0.791	157	0.402	49	0.452	11	0.499
PG 16:0/22:6 (793.5/255.5)	293	0.011	26	0.121	63	0.225	174	0.685	52	0.559	5	0.091
PG 16:1/18:1 (745.5/281.5)	182	0.000	21	0.072	80	0.570	153	0.348	26	0.042	6	0.128
PG 16:1/20:4 (767.4/253.5)	345	0.068	56	0.960	85	0.705	188	0.960	49	0.452	15	1.000
PG 18:1/18:0 (775.6/281.0)	452	0.747	29	0.161	86	0.733	182	0.839	55	0.676	8	0.237
PG 18:1/18:1 (773.4/281.0)	433	0.555	27	0.133	73	0.405	150	0.310	51	0.522	9	0.310
PG 18:1/18:2 (771.8/281.2)	419	0.431	26	0.121	73	0.405	185	0.899	52	0.559	4	0.063
PG 18:1/20:4 (795.6/303.5)	159	0.000	34	0.250	44	0.053	167	0.559	34	0.113	9	0.310
PG 18:1/22.:5 (821.8/281.0)	441	0.633	25	0.109	77	0.495	165	0.526	55	0.676	6	0.128
PG 18:1/22:6 (819.7/281.0)	406	0.332	5	0.009	27	0.010	122	0.084	32	0.090	5	0.091
PG 18:2/22:6 (817.6/279.0)	447	0.694	6	0.011	49	0.081	107	0.035	22	0.024	6	0.128
PG 20:4/22:6 (841.5/303.5)	287	0.008	10	0.019	36	0.025	67	0.002	14	0.007	10	0.398
PG 22:5/22:5 (869.6/329.3)	64	0.000	42	0.453	26	0.009	82	0.006	24	0.032	11	0.499
PG 22:6/22:5 (867.5/329.3)	245	0.001	14	0.031	43	0.049	151	0.322	13	0.006	5	0.091
PG 22:6/22:6 (865.6/327.1)	241	0.001	16	0.040	50	0.088	190	1.000	11	0.004	5	0.091

Table 22 cont....

	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
PI 16:0/18:0 (835.4/283.2)	290	0.009	5	0.009	13	0.002	94	0.015	24	0.032	2	0.028
PI 16:0/20:4 (857.6/255.2)	3	0.000	52	0.802	77	0.495	174	0.685	2	0.001	13	0.735
PI 18:0/18:0 (865.6/283.3)	331	0.043	11	0.021	43	0.049	174	0.685	46	0.359	5	0.091
PI 18:0/18:1 (863.6/283.1)	139	0.000	39	0.368	82	0.622	150	0.310	21	0.021	4	0.063
PI 18:0/20:3 (887.6/283.1)	18	0.000	50	0.726	44	0.053	142	0.223	12	0.005	14	0.866
PI 18:0/20:4 (885.6/283.1)	2	0.000	41	0.423	45	0.058	125	0.099	2	0.001	15	1.000
PI 18:0/22:5 (911.6/283.3)	11	0.000	42	0.453	63	0.225	86	0.008	4	0.001	13	0.735
PI 18:1/18:1 (861.4/281.1)	174	0.000	48	0.652	78	0.520	140	0.204	22	0.024	12	0.612
PI 18:1/20:4 (883.6/281.2)	10	0.000	38	0.342	33	0.019	65	0.002	5	0.001	14	0.866
PS 16:0/16:0 (734.3/255.5)	0	0.000	6	0.011	12	0.002	80	0.005	5	0.001	9	0.310
PS 18:1/18:0 (788.4/283.1)	11	0.000	4	0.008	1	0.000	2	0.000	48	0.420	5	0.091
PE 18:0/20:4 (766.6/303.4)	12	0.000	46	0.582	79	0.544	116	0.060	0	0.001	5	0.091
PE 18:1/18:1 (742.6/281.1)	60	0.000	54	0.881	38	0.031	158	0.417	17	0.011	12	0.612
Total Cer	112	0.000	27	0.133	51	0.096	132	0.141	28	0.055	5	0.091
TOTAL_GC	470	0.944	29	0.161	22	0.006	32	0.000	24	0.032	6	0.128
TOTAL_LC	155	0.000	57	1.000	61	0.198	185	0.899	26	0.042	15	1.000
TOTAL_PC	44	0.000	40	0.395	42	0.045	189	0.980	15	0.008	12	0.612
Total CTH	132	0.000	24	0.099	21	0.005	81	0.006	49	0.452	14	0.866
Total GM3	98	0.000	46	0.582	19	0.004	45	0.000	50	0.487	6	0.128
TOTAL_PG	343	0.064	7	0.012	93	0.940	183	0.859	39	0.191	7	0.176
TOTAL_PI	16	0.000	52	0.802	60	0.185	125	0.099	5	0.001	13	0.735
TOTAL_PS	40	0.000	14	0.031	1	0.000	3	0.000	27	0.048	5	0.091
TOTAL_PE	11	0.000	47	0.617	48	0.075	124	0.094	0	0.001	7	0.176
CTH 16:0 / PC 36:4	0	0.000	10	0.019	18	0.004	110	0.042	1	0.001	7	0.176
PG 18:1_22:6 / PI 18:0_20:4	2	0.000	11	0.021	12	0.002	81	0.006	6	0.002	7	0.176
PI 16:0_18:0 / PI 18:0_20:4	11	0.000	9	0.016	3	0.000	64	0.001	15	0.008	8	0.237
PI 16:0_18:0 / PS 18:1_18:0	14	0.000	0	0.004	0	0.000	1	0.000	34	0.113	12	0.612
PS 16:0_16:0 / PS 18:1_18:0	0	0.000	0	0.004	0	0.000	1	0.000	11	0.004	14	0.866
PG 18:1_22:6 / PG 22:5_22:5	11	0.000	7	0.012	3	0.000	47	0.000	38	0.173	14	0.866

Table 23. Mean lipid concentrations[a] present in urine from control and Fabry patients.

Analyte	Control (n=28)	Hemi (n=13)	Het (n=19)	Hemi (ERT) (n=5)	Het (ERT) (n=10)	Hemi/ Cont	Het/ Cont
Cer C16:0 (538.7/264.4)	20	31	37	55	24	1.6	1.9
Cer C24:0 (650.7/264.4)	12	18	16	40	11	1.5	1.3
Cer C24:1 (648.7/264.4)	5	14	11	21	5	2.7	2.2
Cer C20:0 (592.7/264.4)	2	2	2	8	1	1.0	0.8
Cer C20:1 (590.7/264.4)	3	2	12	15	3	0.9	4.3
Cer C23:0 (636.7/264.4)	5	5	23	37	4	1.1	4.6
Cer C23:1 (634.8/264.4)	4	5	8	20	5	1.2	2.0
GC C16:0 (700.6/264.4)	28	25	25	73	22	0.9	0.9
GC C22:0 (784.7/264.4)	38	32	23	75	21	0.8	0.6
GC C24:0 (812.7/264.4)	34	30	25	66	22	0.9	0.7
GC C24:1 (810.8/264.4)	12	14	10	45	9	1.2	0.8
LC C16:0 (862.4/264.4)	158	386	336	556	451	2.4	2.1
LC C20:0 (918.7/264.4)	118	317	138	614	185	2.7	1.2
LC C22:0 (946.7/264.4)	111	406	178	682	263	3.7	1.6
LC C22:0-OH (962.7/264.4)	147	681	203	843	334	4.6	1.4
LC C24:0 (974.8/264.4)	94	727	176	529	255	7.7	1.9
LC C24:1 (972.8/264.4)	86	311	202	498	238	3.6	2.4
(LC) CTH C16:0 (1024.8/264.4)	70	998	151	1288	293	14.4	2.2
(LC) CTH C18:0 (1052.7/264.4)	46	505	83	520	97	11.0	1.8
(LC) CTH C20:0 (1080.9/264.4)	186	817	162	1008	194	4.4	0.9
(LC) CTH C22:0 (1108.9/264.4)	75	1964	213	1791	350	26.1	2.8
(LC) CTH C24:0 (1136.9/264.4)	74	2669	178	2361	486	35.9	2.4
(LC) CTH C24:1 (1134.9/264.4)	85	2124	237	1586	389	25.0	2.8
PC C32:0 (734.7/184.1)	57	44	51	57	46	0.8	0.9
PC C32:1 (732.7/184.1)	57	40	54	54	47	0.7	1.0
PC C34:1 (760.6/184.1)	397	353	344	382	338	0.9	0.9
PC C34:2 (758.5/184.1)	219	261	189	201	241	1.2	0.9
PC C36:2 (786.6/184.1)	163	171	195	193	184	1.0	1.2
PC C36:4 (782.6/184.1)	78	95	123	81	106	1.2	1.6
PC C38:4 (810.8/184.1)	30	37	44	31	39	1.2	1.5
SM C16:0 (703.9/184.1)	209	199	261	246	175	1.0	1.2
SM C22:0 (787.8/184.1)	293	215	267	249	175	0.7	0.9
SM C24:0 (815.8/184.1)	245	161	175	196	132	0.7	0.7

Table 23 cont....

Analyte	Control (n=28)	Hemi (n=13)	Het (n=19)	Hemi (ERT) (n=5)	Het (ERT) (n=10)	Hemi/ Cont	Het/ Cont
PG 16:0/18:1 (747.6/255.8)	0	2	1	1	1	4.8	2.5
PG 16:0/22:6 (793.5/255.5)	2	12	4	10	3	4.9	1.7
PG 16:1/18:1 (745.5/281.5)	2	7	9	5	3	3.6	5.0
PG 16:1/20:4 (767.4/253.5)	1	1	1	1	0	1.1	1.5
PG 18:1/18:0 (775.6/281.0)	5	43	20	24	16	8.5	3.9
PG 18:1/18:1 (773.4/281.0)	21	264	63	132	80	12.7	3.0
PG 18:1/18:2 (771.8/281.2)	5	83	17	37	24	15.2	3.1
PG 18:1/20:4 (795.6/303.5)	1	6	4	6	3	7.5	4.6
PG 18:1/22.:5 (821.8/281.0)	3	24	7	12	7	9.6	2.9
PG 18:1/22:6 (819.7/281.0)	10	93	16	68	25	9.3	1.6
PG 18:2/22:6 (817.6/279.0)	5	40	8	36	13	7.4	1.5
PG 20:4/22:6 (841.5/303.5)	1	3	1	4	1	4.1	1.3
PG 22:5/22:5 (869.6/329.3)	0	3	1	1	1	6.7	1.3
PG 22:6/22:5 (867.5/329.3)	1	6	2	7	3	4.8	1.2
PG 22:6/22:6 (865.6/327.1)	4	18	4	23	7	4.8	1.1
PI 16:0/18:0 (835.4/283.2)	33	27	23	30	18	0.8	0.7
PI 16:0/20:4 (857.6/255.2)	13	11	10	11	5	0.9	0.8
PI 18:0/18:0 (865.6/283.3)	19	82	14	103	20	4.4	0.7
PI 18:0/18:1 (863.6/283.1)	17	16	35	17	8	0.9	2.0
PI 18:0/20:3 (887.6/283.1)	19	18	30	15	8	0.9	1.6
PI 18:0/20:4 (885.6/283.1)	64	61	102	52	32	1.0	1.6
PI 18:0/22:5 (911.6/283.3)	4	4	6	4	2	1.0	1.6
PI 18:1/18:1 (861.4/281.1)	9	9	69	8	4	1.0	7.8
PI 18:1/20:4 (883.6/281.2)	7	6	10	6	3	0.9	1.4
PS 16:0/16:0 (734.3/255.5)	1	1	62	3	43	1.2	69.5
PS 18:1/18:0 (788.4/283.1)	72	38	50	40	30	0.5	0.7
PE 18:0/20:4 (766.6/303.4)	3	3	3	3	2	0.8	1.0
PE 18:1/18:1 (742.6/281.1)	8	6	14	8	5	0.7	1.7
total Cer	51	78	110	196	53	1.5	2.2
total GC	112	101	83	259	74	0.9	0.7
total LC	714	2829	1232	3722	1726	4.0	1.7
total CTH	536	9078	1024	8554	1808	17.0	1.9
total PC	1000	1000	1000	1000	1000	1.0	1.0
total SM	748	576	703	691	481	0.8	0.9
total PG	61	604	156	366	187	9.8	2.5
total PI	152	208	276	215	80	1.4	1.8
total PE	11	9	17	12	7	0.8	1.5
total PS	73	39	111	42	73	0.5	1.5

[a] Determination of lipid species was semi-quantitative (see Results and Discussion). Results are expressed as pmol/nmol total PC

Table 24. Statistical analysis of lipid levels in urine samples from control, Fabry hemizygotes, Fabry heterozygotes, Fabry hemizygotes on ERT and Fabry heterozygotes on ERT.
Control (n=28); Hemi (n=13); Het (n=19); Hemi (ERT) (n=5); Het (ERT) (N=10)

Analyte	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
Cer C16:0 (538.7/264.4)	110	0.044	193	0.114	11	0.003	108	0.289	14	0.068	88	0.748
Cer C24:0 (650.7/264.4)	111	0.047	236	0.515	9	0.002	140	1.000	9	0.021	82	0.551
Cer C24:1 (648.7/264.4)	70	0.002	239	0.558	8	0.002	133	0.817	18	0.153	87	0.714
Cer C20:0 (592.7/264.4)	151	0.385	232	0.461	45	0.209	108	0.289	28	0.657	83	0.582
Cer C20:1 (590.7/264.4)	181	0.978	178	0.056	60	0.616	135	0.868	27	0.588	58	0.090
Cer C23:0 (636.7/264.4)	132	0.161	241	0.588	32	0.056	114	0.389	24	0.402	93	0.927
Cer C23:1 (634.8/264.4)	118	0.073	219	0.308	48	0.269	121	0.529	31	0.882	68	0.215
GC C16:0 (700.6/264.4)	161	0.556	237	0.530	35	0.079	112	0.353	14	0.068	87	0.714
GC C22:0 (784.7/264.4)	129	0.138	113	0.001	43	0.175	43	0.001	14	0.068	90	0.819
GC C24:0 (812.7/264.4)	161	0.556	163	0.026	37	0.098	63	0.011	13	0.055	94	0.963
GC C24:1 (810.8/264.4)	143	0.275	199	0.146	36	0.088	92	0.112	23	0.349	94	0.963
LC C16:0 (862.4/264.4)	101	0.023	131	0.003	7	0.002	52	0.004	22	0.301	85	0.646
LC C20:0 (918.7/264.4)	47	0.000	247	0.680	9	0.002	112	0.353	22	0.301	80	0.491
LC C22:0 (946.7/264.4)	49	0.000	175	0.049	2	0.001	71	0.022	19	0.183	82	0.551
LC C22:0-OH (962.7/264.4)	58	0.001	212	0.242	6	0.001	110	0.320	20	0.218	85	0.646
LC C24:0 (974.8/264.4)	9	0.000	123	0.002	1	0.001	63	0.011	29	0.730	82	0.551
LC C24:1 (972.8/264.4)	43	0.000	105	0.000	3	0.001	47	0.002	23	0.349	91	0.854
(LC) CTH C16:0 (1024.8/264.4)	35	0.000	160	0.022	3	0.001	57	0.006	17	0.127	82	0.551
(LC) CTH C18:0 (1052.7/264.4)	21	0.000	193	0.114	17	0.008	112	0.353	32	0.961	92	0.891
(LC) CTH C20:0 (1080.9/264.4)	67	0.001	244	0.633	15	0.006	134	0.842	20	0.218	92	0.891
(LC) CTH C22:0 (1108.9/264.4)	39	0.000	134	0.004	6	0.001	92	0.112	30	0.805	85	0.646
(LC) CTH C24:0 (1136.9/264.4)	23	0.000	138	0.006	4	0.001	70	0.020	30	0.805	94	0.963
(LC) CTH C24:1 (1134.9/264.4)	42	0.000	126	0.002	3	0.001	69	0.019	32	0.961	94	0.963

Table 24 cont....

Analyte	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
PC C32:0 (734.7/184.1)	98	0.019	239	0.558	68	0.920	106	0.260	10	0.027	84	0.614
PC C32:1 (732.7/184.1)	51	0.000	263	0.948	55	0.451	81	0.050	7	0.012	62	0.130
PC C34:1 (760.6/184.1)	97	0.017	145	0.009	55	0.451	51	0.003	22	0.301	77	0.409
PC C34:2 (758.5/184.1)	74	0.002	211	0.233	58	0.547	98	0.164	7	0.012	52	0.048
PC C36:2 (786.6/184.1)	133	0.170	145	0.009	68	0.920	72	0.024	19	0.183	74	0.335
PC C36:4 (782.6/184.1)	82	0.005	218	0.298	49	0.292	61	0.009	21	0.257	43	0.017
PC C38:4 (810.8/184.1)	60	0.001	210	0.225	42	0.160	70	0.020	22	0.301	42	0.015
SM C16:0 (703.9/184.1)	168	0.695	240	0.573	40	0.132	94	0.127	18	0.153	76	0.383
SM C22:0 (787.8/184.1)	71	0.002	137	0.005	49	0.292	21	0.000	17	0.127	69	0.233
SM C24:0 (815.8/184.1)	57	0.000	118	0.001	42	0.160	26	0.000	18	0.153	81	0.521
PG 16:0/18:1 (747.6/255.8)	67	0.001	118	0.001	33	0.063	102	0.208	24	0.402	77	0.409
PG 16:0/22:6 (793.5/255.5)	56	0.000	192	0.109	37	0.098	134	0.842	24	0.402	79	0.463
PG 16:1/18:1 (745.5/281.5)	68	0.001	84	0.000	33	0.063	117	0.446	25	0.460	77	0.409
PG 16:1/20:4 (767.4/253.5)	132	0.161	252	0.762	52	0.366	102	0.208	25	0.460	72	0.291
PG 18:1/18:0 (775.6/281.0)	23	0.000	89	0.000	21	0.014	103	0.220	23	0.349	81	0.521
PG 18:1/18:1 (773.4/281.0)	12	0.000	96	0.000	10	0.003	96	0.145	21	0.257	86	0.680
PG 18:1/18:2 (771.8/281.2)	4	0.000	83	0.000	12	0.004	82	0.055	17	0.127	88	0.748
PG 18:1/20:4 (795.6/303.5)	20	0.000	168	0.034	20	0.012	110	0.320	31	0.882	93	0.927
PG 18:1/22.:5 (821.8/281.0)	10	0.000	154	0.015	27	0.031	112	0.353	21	0.257	90	0.819
PG 18:1/22:6 (819.7/281.0)	24	0.000	188	0.091	24	0.021	105	0.246	19	0.183	88	0.748
PG 18:2/22:6 (817.6/279.0)	23	0.000	201	0.159	24	0.021	104	0.233	21	0.257	88	0.748
PG 20:4/22:6 (841.5/303.5)	26	0.000	242	0.603	32	0.056	113	0.371	29	0.730	89	0.783
PG 22:5/22:5 (869.6/329.3)	25	0.000	245	0.649	42	0.160	127	0.667	24	0.402	92	0.891
PG 22:6/22:5 (867.5/329.3)	31	0.000	259	0.879	38	0.108	106	0.260	20	0.218	78	0.435
PG 22:6/22:6 (865.6/327.1)	36	0.000	252	0.762	41	0.145	117	0.446	20	0.218	87	0.714

Table 24 cont....

Analyte	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
PI 16:0/18:0 (835.4/283.2)	134	0.179	252	0.762	51	0.340	84	0.063	27	0.588	68	0.215
PI 16:0/20:4 (857.6/255.2)	159	0.519	172	0.042	63	0.725	37	0.001	31	0.882	71	0.271
PI 18:0/18:0 (865.6/283.3)	53	0.000	197	0.135	35	0.079	124	0.596	28	0.657	84	0.614
PI 18:0/18:1 (863.6/283.1)	178	0.911	211	0.233	63	0.725	23	0.000	24	0.402	25	0.001
PI 18:0/20:3 (887.6/283.1)	175	0.845	161	0.023	57	0.514	24	0.000	28	0.657	51	0.044
PI 18:0/20:4 (885.6/283.1)	169	0.716	144	0.008	58	0.547	36	0.001	26	0.522	74	0.335
PI 18:0/22:5 (911.6/283.3)	160	0.538	185	0.079	65	0.802	49	0.003	28	0.657	70	0.251
PI 18:1/18:1 (861.4/281.1)	137	0.207	226	0.386	59	0.581	46	0.002	28	0.657	50	0.039
PI 18:1/20:4 (883.6/281.2)	169	0.716	183	0.072	65	0.802	24	0.000	29	0.730	46	0.025
PS 16:0/16:0 (734.3/255.5)	122	0.093	234	0.488	58	0.547	125	0.619	29	0.730	93	0.927
PS 18:1/18:0 (788.4/283.1)	71	0.002	126	0.002	37	0.098	33	0.000	29	0.730	69	0.233
PE 18:0/20:4 (766.6/303.4)	149	0.355	184	0.075	67	0.880	92	0.112	29	0.730	94	0.963
PE 18:1/18:1 (742.6/281.1)	139	0.228	257	0.845	66	0.841	70	0.020	26	0.522	48	0.031
total Cer	109	0.041	204	0.179	7	0.002	135	0.868	13	0.055	76	0.383
total GC	165	0.634	164	0.027	36	0.088	63	0.011	13	0.055	89	0.783
total LC	29	0.000	139	0.006	4	0.001	56	0.005	25	0.460	85	0.646
total CTH	38	0.000	159	0.020	4	0.001	90	0.097	32	0.961	95	1.000
total PC	182	1.000	266	1.000	70	1.000	140	1.000	32.5	1.000	95	1.000
total SM	81	0.005	170	0.037	56	0.482	36	0.001	16	0.104	78	0.435
total PG	13	0.000	129	0.003	19	0.010	106	0.260	22	0.301	87	0.714
total PI	103	0.027	166	0.030	54	0.422	46	0.002	27	0.588	67	0.199
total PE	139	0.228	242	0.603	63	0.725	78	0.040	27	0.588	58	0.090
total PS	73	0.002	152	0.013	39	0.120	56	0.005	29	0.730	72	0.291

Table 24 cont....

Analyte	Cont vs Hemi		Cont vs Het		Cont vs Hemi (ERT)		Cont vs Het (ERT)		Hemi vs Hemi (ERT)		Het vs Het (ERT)	
	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.	M-W U	Sig.
CTH24:0/SM24:0	11	0.000	88	0.000	2	0.001	9	0.000	25	0.460	82	0.551
Cer24:1/GC22:0	42	0.000	76	0.000	13	0.004	23	0.000	29	0.730	93	0.927
LC24:0/GC22:0	1	0.000	49	0.000	0	0.000	0	0.000	12	0.043	64	0.155
PG18:1/18:1/SM24:0	4	0.000	22	0.000	12	0.004	43	0.001	20	0.218	83	0.582
PG18:1/18:1 /PS 18:1/18:0	2	0.000	34	0.000	1	0.001	7	0.000	23	0.349	75	0.359
PI18:0/18:0 / PS 18:1/18:0	29	0.000	219	0.308	27	0.031	35	0.001	29	0.730	37	0.008
PC38:4/PC32:1	55	0.000	243	0.618	43	0.175	47	0.002	16	0.104	39	0.010
CTH24:0*PG18:1/18:1/SM24:0	6	0.000	65	0.000	1	0.001	40	0.001	27	0.588	92	0.891
PG18:1/18:1* PI18:0/18:0 / PS 18:1/18:0	13	0.000	117	0.001	2	0.001	37	0.001	26	0.522	80	0.491
CTH24:0*LC24:0/GC22:0/SM24:0	3	0.000	65	0.000	0	0.000	1	0.000	20	0.218	71	0.271
CTH24:0*LC24:0*Cer24:1/GC22:0/SM24:0	9	0.000	50	0.000	0	0.000	7	0.000	26	0.522	81	0.521
PC38:4*PG18:1/18:1* PI18:0/18:0 / PC32:1/PS 18:1/18:0	4	0.000	134	0.004	2	0.001	30	0.000	24	0.402	69	0.233
CTH24:0*LC24:0/PS18:1/18:0	9	0.000	90	0.000	0	0.000	8	0.000	32	0.961	75	0.359
PG18:1/18:1/GC22:0/SM24:0	2	0.000	12	0.000	28	0.035	29	0.000	12	0.043	86	0.680
PG18:1/18:2/GC22:0/SM24:0	0	0.000	7	0.000	50	0.315	25	0.000	12	0.043	78	0.435
CTH22:0*LC24:0/PS18:1/18:0	7	0.000	67	0.000	1	0.001	13	0.000	31	0.882	81	0.521

REFERENCES

1. Meikle, P.J., Hopwood, J.J., Clague, A.E. and Carey, W.F., Prevalence of lysosomal storage disorders. *Jama.* 1999, **281**: 249-254.

2. Rider, J.A. and Rider, D.L., Thirty years of Batten disease research: present status and future goals. *Mol. Genet. Metab.* 1999, **66**: 231-233.

3. Santavuori, P., Neuronal ceroid-lipofuscinoses in childhood. *Brain Dev.* 1988, **10**: 80-83.

4. Conzelmann, E. and Sandhoff, K., Partial enzyme deficiencies: residual activities and the development of neurological disorders. *Dev. Neurosci.* 1983, **6**: 58-71.

5. Leinekugel, P., Michel, S., Conzelmann, E. and Sandhoff, K., Quantitative correlation between the residual activity of beta-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. *Hum. Genet.* 1992, **88**: 513-523.

6. Carpenter, K.H. and Wiley, V., Application of tandem mass spectrometry to biochemical genetics and newborn screening. *Clin. Chim. Acta.* 2002, **322**: 1-10.

7. Chace, D.H., Kalas, T.A. and Naylor, E.W., The application of tandem mass spectrometry to neonatal screening for inherited disorders of intermediary metabolism. *Annu. Rev. Genomics Hum. Genet.* 2002, **3**: 17-45.

CLAIMS

1. A method of assessing an LSD (Lysosomal storage disorder) status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of three or more compound indicators, said indicators being indicative of the level of respectively each of three or more lipid containing storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual.

2. A method of assessing an LSD status of an individual the method comprising the steps of,

taking a tissue or body fluid sample from the individual,

estimating a level in the sample of each of two or more compound indicators being indicative of the level respectively of each of two or more lipid containing storage associated compounds,

calculating an LSD index number using all of said compound indicators,

and comparing the LSD index number of the sample with a standard to provide an assessment of the LSD status of the individual,

the two or more storage associated compounds selected to discriminate between an LSD individual from a non-LSD individual with an acceptable confidence level.

3. A method for screening for the status of two or more LSDs in an individual,

taking a single tissue or body fluid sample from the individual,

estimating a level in the sample of each three or more compound indicators being indicative of the concentration respectively of each of three or more lipid containing storage associated compounds,

calculating a first LSD index number using a first set of two or more of said compound indicators and comparing the first LSD index number of the sample with a first control indicator to provide an assessment of the LSD status of the first LSD,

and calculating a second LSD index number using a second set of two or more of said compound indicators and comparing the second LSD index number of the individual with a second standard to provide an assessment of the LSD status of the second LSD in the individual.

4. The method as in any one of claims 1 to 3 wherein the storage associated compounds are selected from the group of compounds consisting of phospholipids and glycolipids.

5. The method of claim 4 wherein the glycolipids are selected from the group comprising glycerolipids, glycoposhatidylinositols, glycosphingolipids.

6. The method of claim 4 wherein the storage associated compounds are phospholipids and are characterised by head groups selected from the group consisting of phosphatidyl serine, phosphatidylinositol, phosphatidyl ethanolamine and sphingomyelin phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol, phosophatidyl ethanolamine, cerebroside or a ganglioside.

7. The method of claim 6 wherein the phospholipids are further characterised by the fatty acids which are selected from the group consisting of 1-palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl -, 1-palmitoly-2-arachadonyl -, 1-palmitoyl-2-docosahexanoyl.

8. The method of claim 4 wherein the indicator of the level of lipid containing storage associated compound is measured by a technique selected from the group consisting of electrophoresis, chromatography, Gas chromatography, HPLC (High pressure Liquid Chromatography), Nuclear Magnetic resonance analysis, gas chromatography-mass spectrometry (GC-MS), GC linked to Fourier-transform infrared spectroscopy (FTIR), and silver ion and reversed-phase high-performance liquid chromatography (HPLC) and mass spectrometry.

9. The method of claim 8 wherein the technique is mass spectrometry.

10. The method of claim 9 where the mass sepctrometry is electrospray ionisation-tandem mass spectrometry (ESI-MSMS).

11. The method as in claim 4 wherein at least two lipid containing storage associated compounds are selected one from a first group that increases in LSD individual and a second from a second group that decreases in levels in LSD individual and the values for the first and second compounds are combined to give an index number.

12. The method as in claim 4 wherein the is whole blood or products derived therefrom.

13. The method as in claim 4 wherein the samples are obtained from young patients selected from the group consisting of embryos, foetuses, neonatals, young infants.

14. The method of claim 4 used to determine subclinical levels of the LSD before onset of physical manifestations become apparent.

15. The method of claim 4 wherein the LSD is Gaucher disease.

16. The method of claim 3 to measure the severity of the LSD.

17. The method of either claim 1 or 3 wherein the LSD is Fabry and a first compound is selected from the group consisting of Cer (ceramide), LC (lactosyl ceramide), CTH (trihexosyceramide) and the second compound is selected from the group consisting of SM (sphingomyelin) and GC (glucosylceramide).

18. The method of claim 17 wherein two or more of Cer, LC and CTH is compared to SM.

19. The method of claim 17 wherein two or more of Cer, LC and CTH is compared to GC.

20. The method of claim 17 wherein the Cer, LC and CTH are C24:1 species.

21. The method of claim 20 wherein CTH and LC (24:1) is compared to SM (C24:0).

22. The method of claim 17 wherein the index is calculated according to the following calculation (LC C24:1*CTH C24:1)/(GC C24:0*SM C24:0).

23. The method of either claim 1 or 3 wherein the LSD is Gaucher and two compounds are selected from the group consisting of SM, LC CTH and the third compound is selected from the group consisting of Cer and GC.

24. The method of claim 23 wherein two or more of SM, LC and CTH are compared to Cer.

25. The method of claim 23 wherein two or more of SM LC and CTH are compared with GC.

26. The method of claim 23 wherein two or more of SM LC and CTH are compared with Cer and GC.

27. A method of developing a diagnostic method comprising the steps of

taking a first group of LSD samples one each from a plurality of LSD individuals affected by one type of LSD,

taking a second group of control samples one each from a plurality of control individuals not affected by LSD

the sample being of a tissue or body fluid of the individual

an LSD group of individuals with LSD

interrogating the first group of samples by mass spectrometry for first levels of a plurality of indicators of respective lipid containing storage associated compounds,

interrogating the second group of samples by mass spectrometry for second levels of the plurality of indicators of respective lipid containing storage associated compounds,

the lipid containing storage associated compounds selected from the class of compounds consisting of the group glycolipids and phospholipids,

comparing the first levels with the second levels

identifying a first group of lipid containing storage associated compound which are shown as having increased levels of indicators in the first LSD group compared to the control group,

identifying a second group of lipid containing storage associated compounds which are shows as having decreased levels of indicators in the LSD group compared to the control group,

formulating a combination of two or more of the first and/or second group of indicators by which to calculate and index number whereby to distinguish LSD samples from control samples, and preferably

preparing a standard being a scale of index numbers reflective of the severity of the LSD.

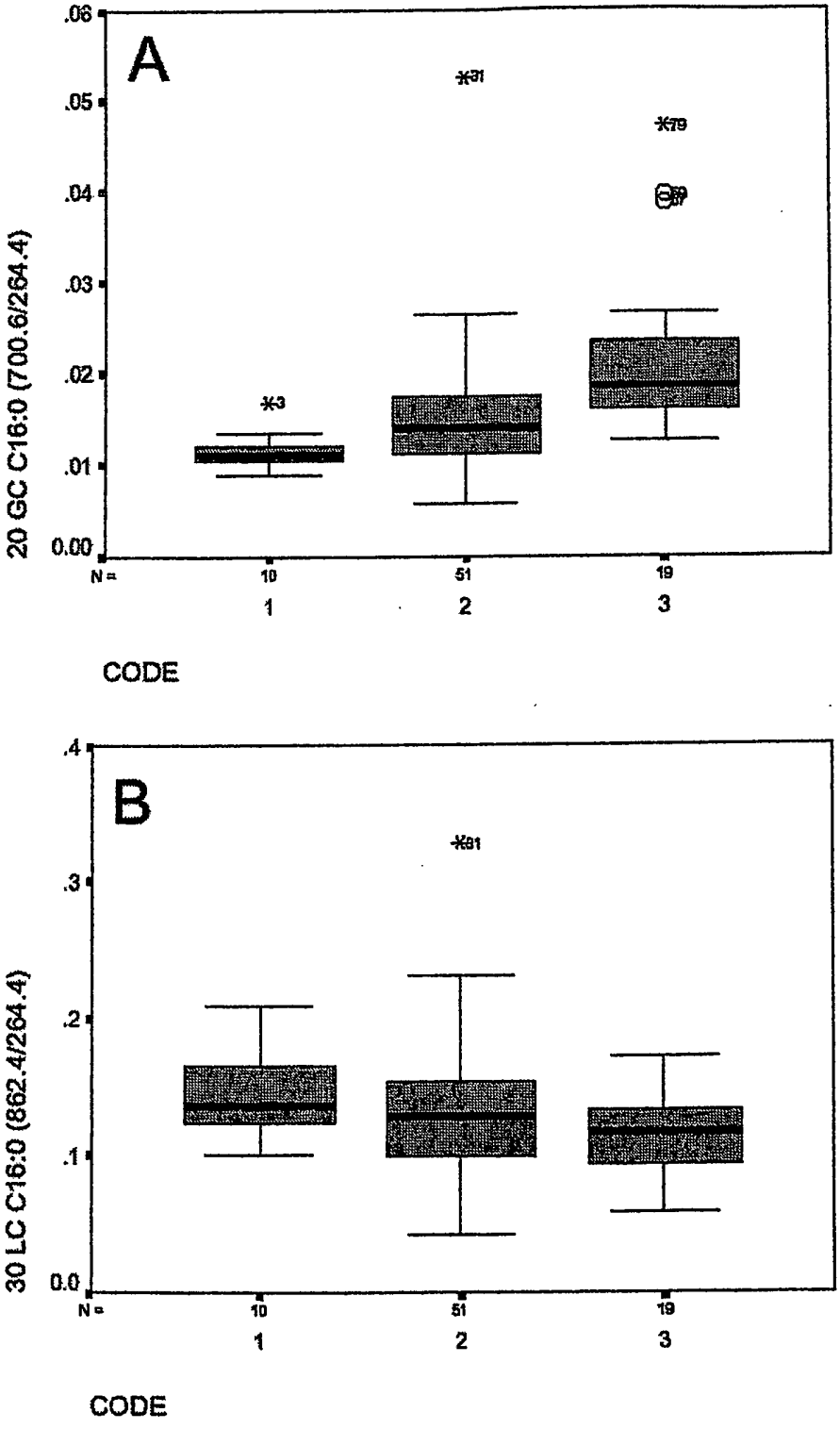
A
20 GC C16:0 (700.6/264.4)
.06
.05
.04
.03
.02
.01
0.00
N =
10
51
19
1
2
3
CODE
B
30 LC C16:0 (862.4/264.4)
.4
.3
.2
.1
0.0
N =
10
51
19
1
2
3
CODE

FIGURE 1

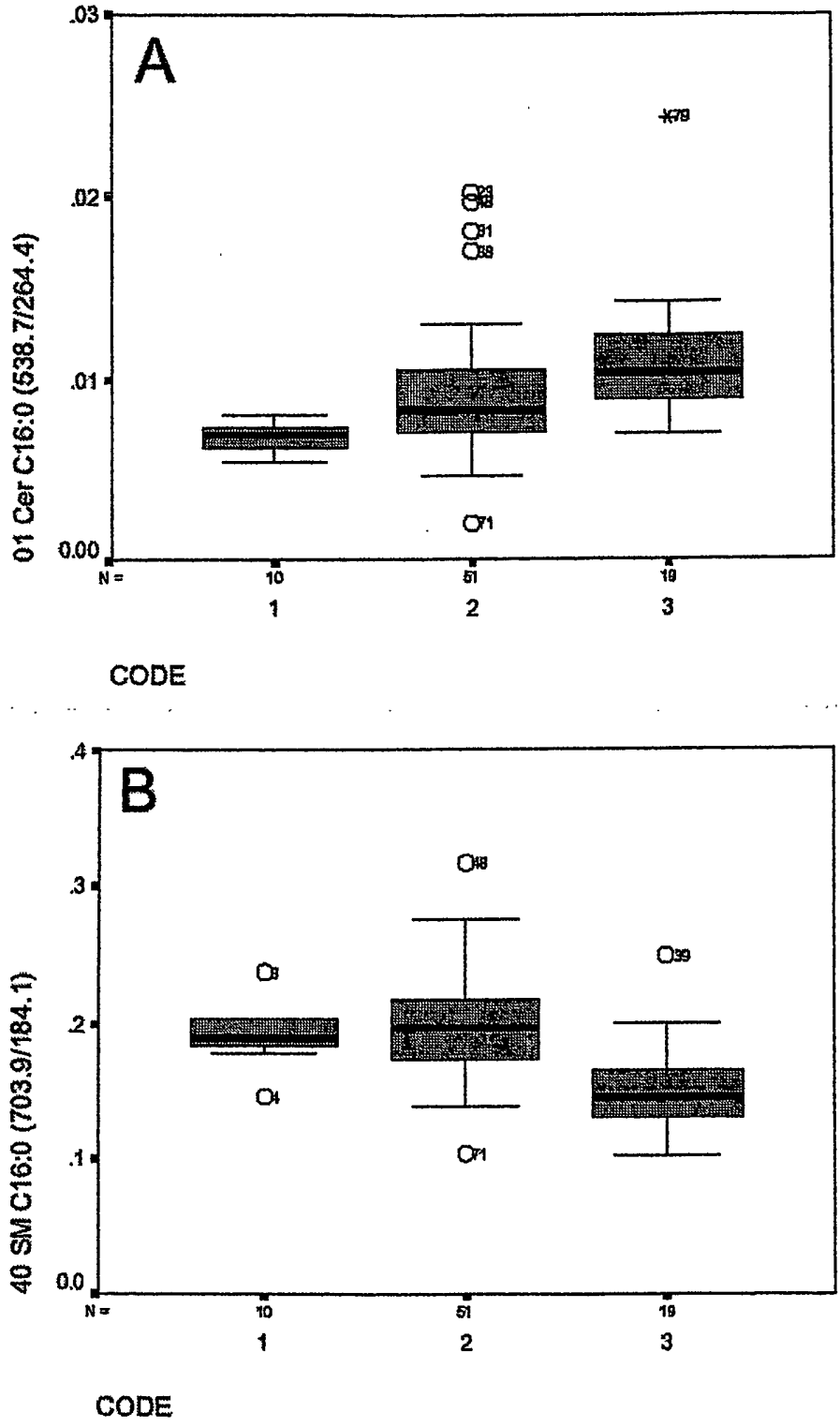
A
01 Cer C16:0 (538.7/264.4)
.03
.02
.01
0.00
N =
10
51
19
1
2
3
CODE
B
40 SM C16:0 (703.9/184.1)
.4
.3
.2
.1
0.0
N =
10
51
19
1
2
3
CODE

FIGURE 2

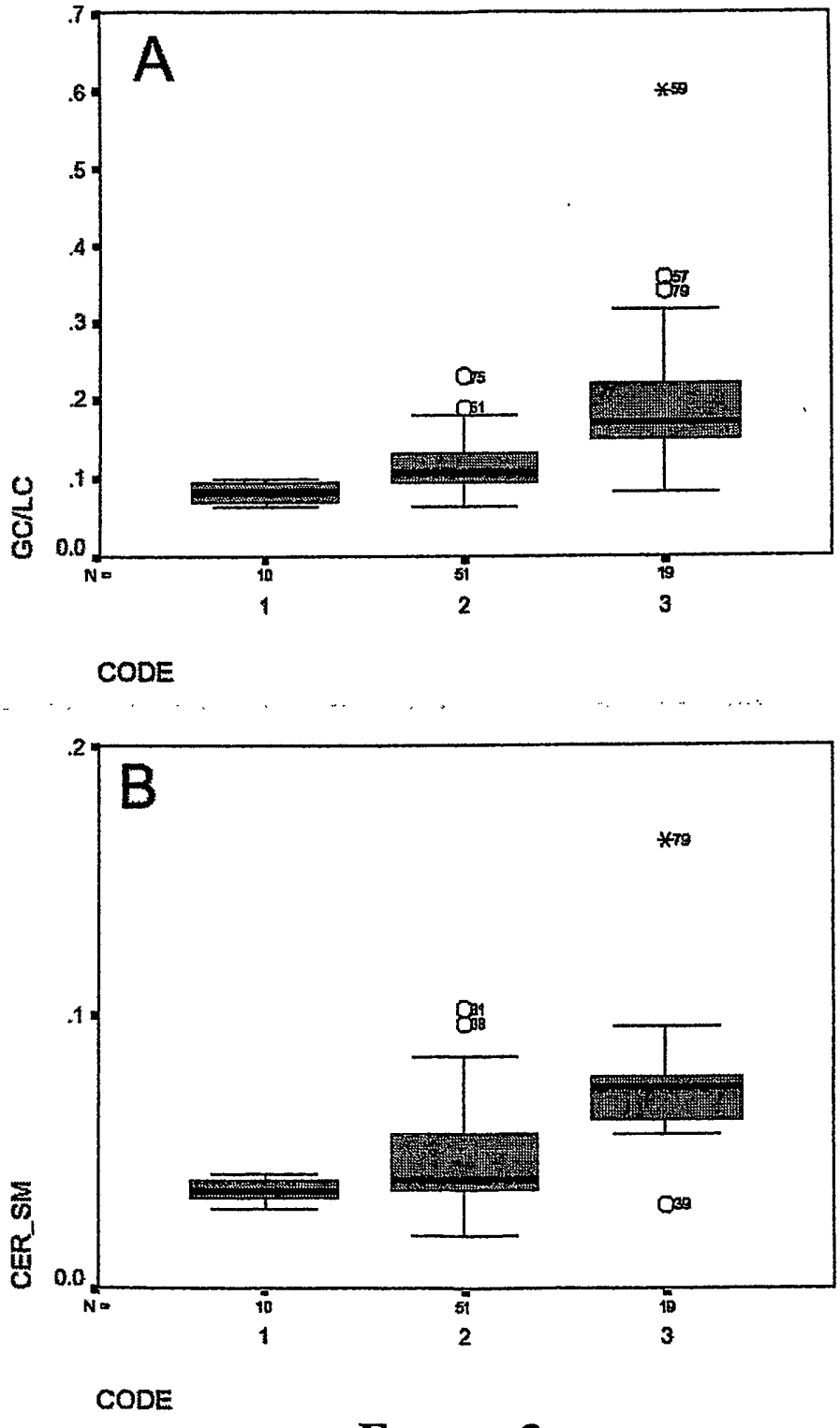
A
GC/LC
.7
.6
.5
.4
.3
.2
.1
0.0
59
57
79
75
61
N =
10
51
19
1
2
3
CODE
B
CER_SM
.2
.1
0.0
79
81
68
39
N =
10
51
19
1
2
3
CODE

FIGURE 3

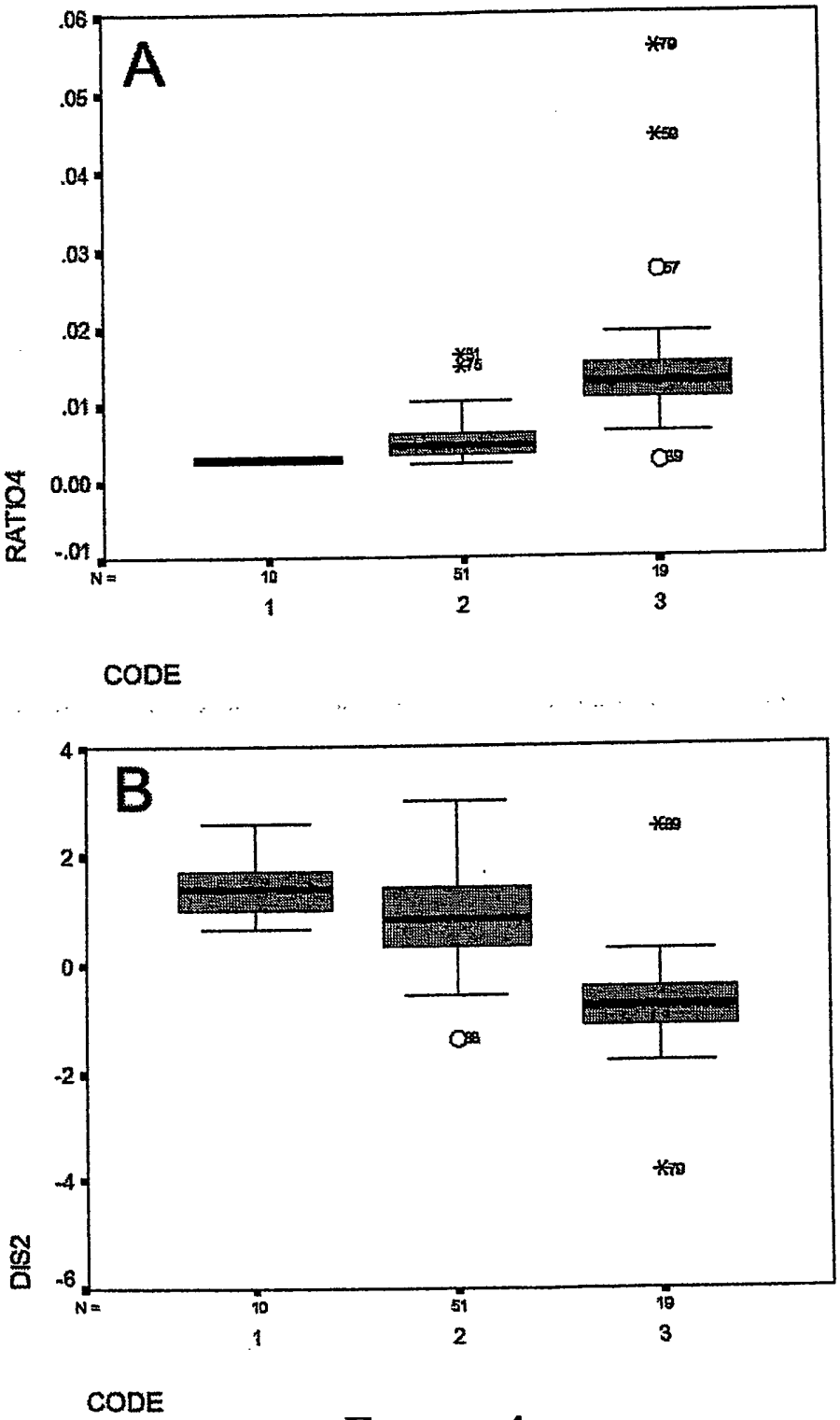
A
.06
.05
.04
.03
.02
.01
0.00
-.01
RATIO4
N =
10
51
19
1
2
3
CODE
B
4
2
0
-2
-4
-6
DIS2
N =
10
51
19
1
2
3
CODE

FIGURE 4

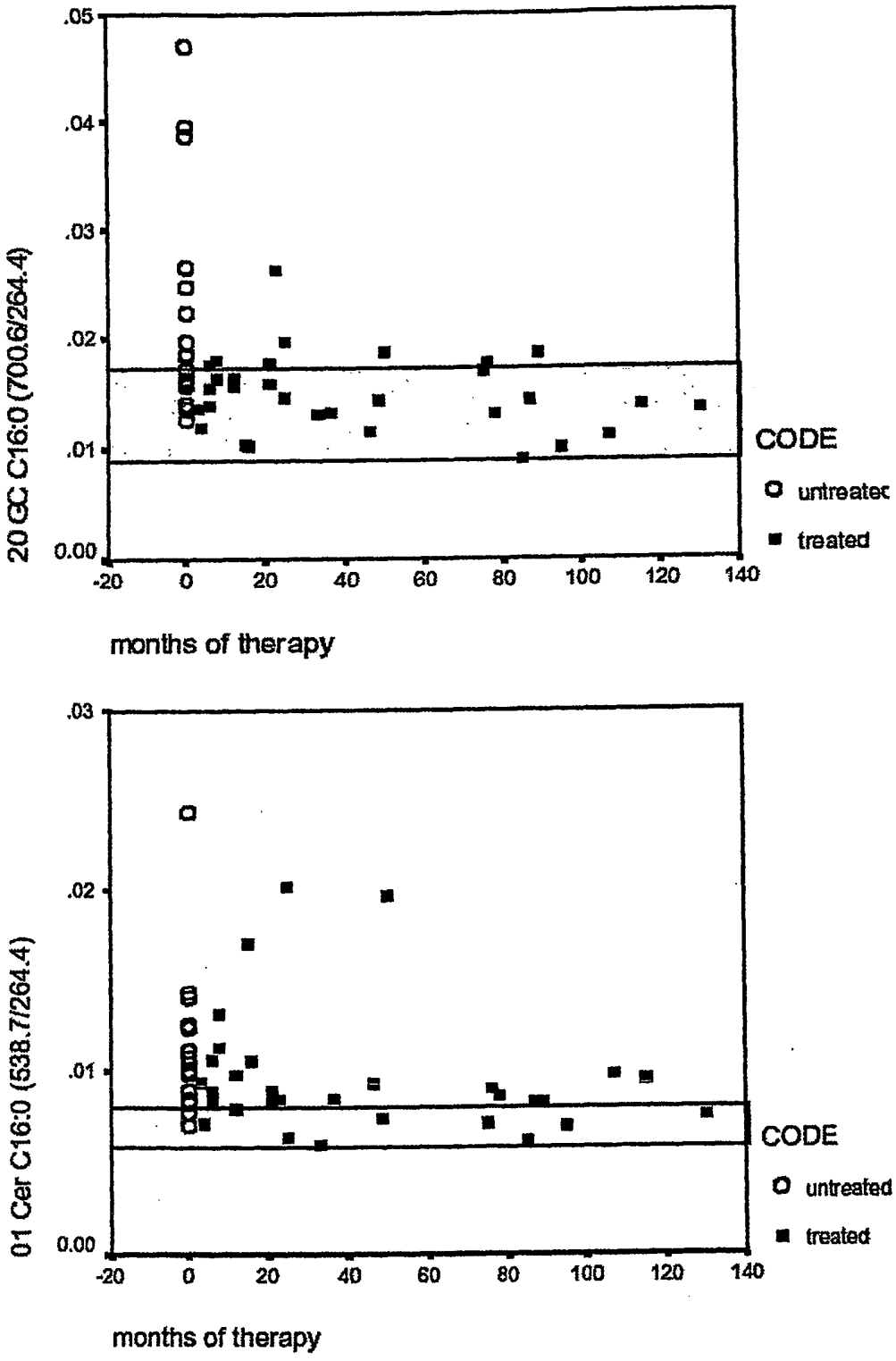
.05
.04
.03
.02
.01
0.00
20 GC C16:0 (700.6/264.4)
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated
.03
.02
.01
0.00
01 Cer C16:0 (538.7/264.4)
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated

FIGURE 5

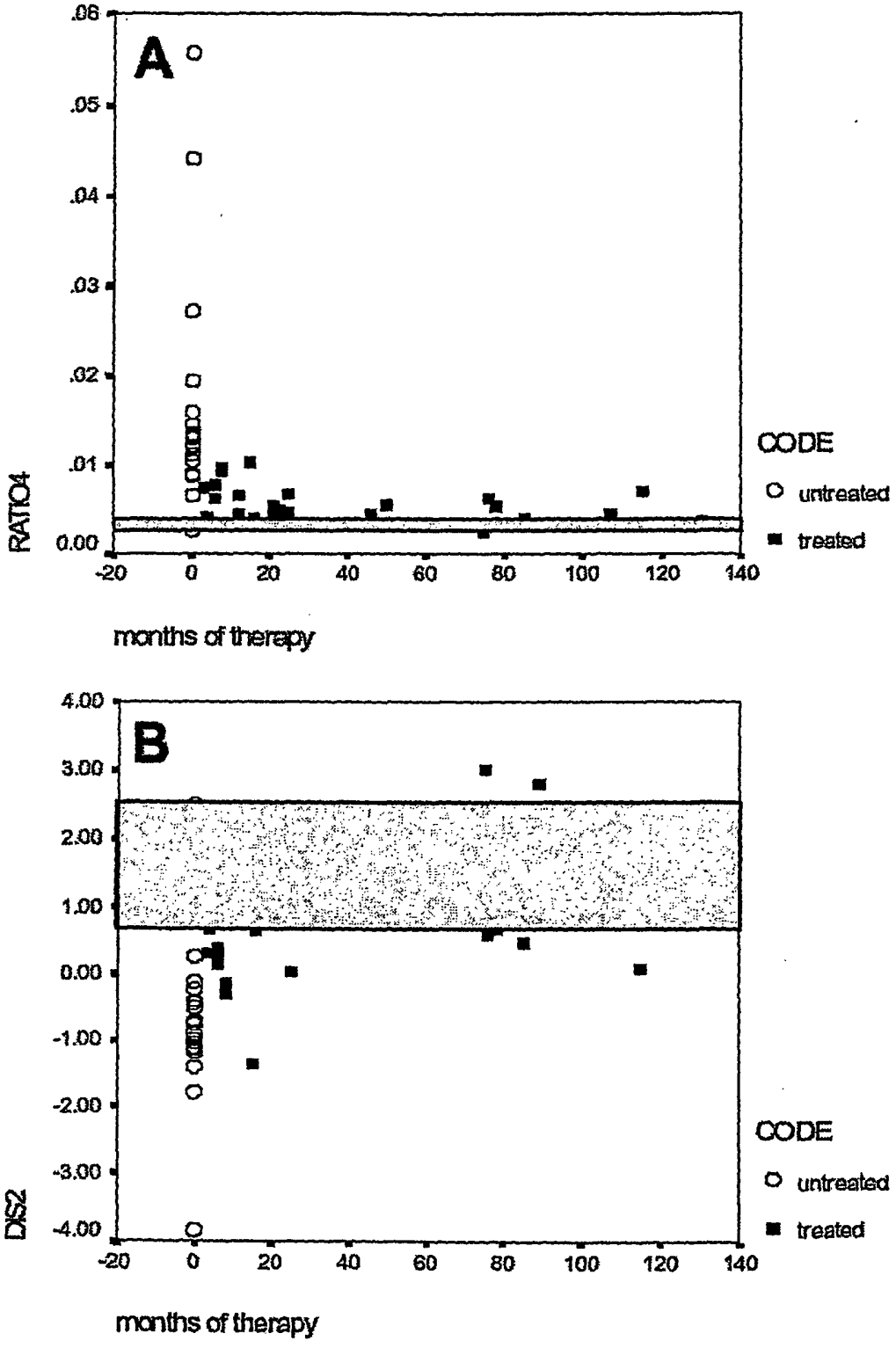
A
.06
.05
.04
.03
.02
.01
0.00
RATIO4
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated
B
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
DIS2
-20
0
20
40
60
80
100
120
140
months of therapy
CODE
untreated
treated

FIGURE 6

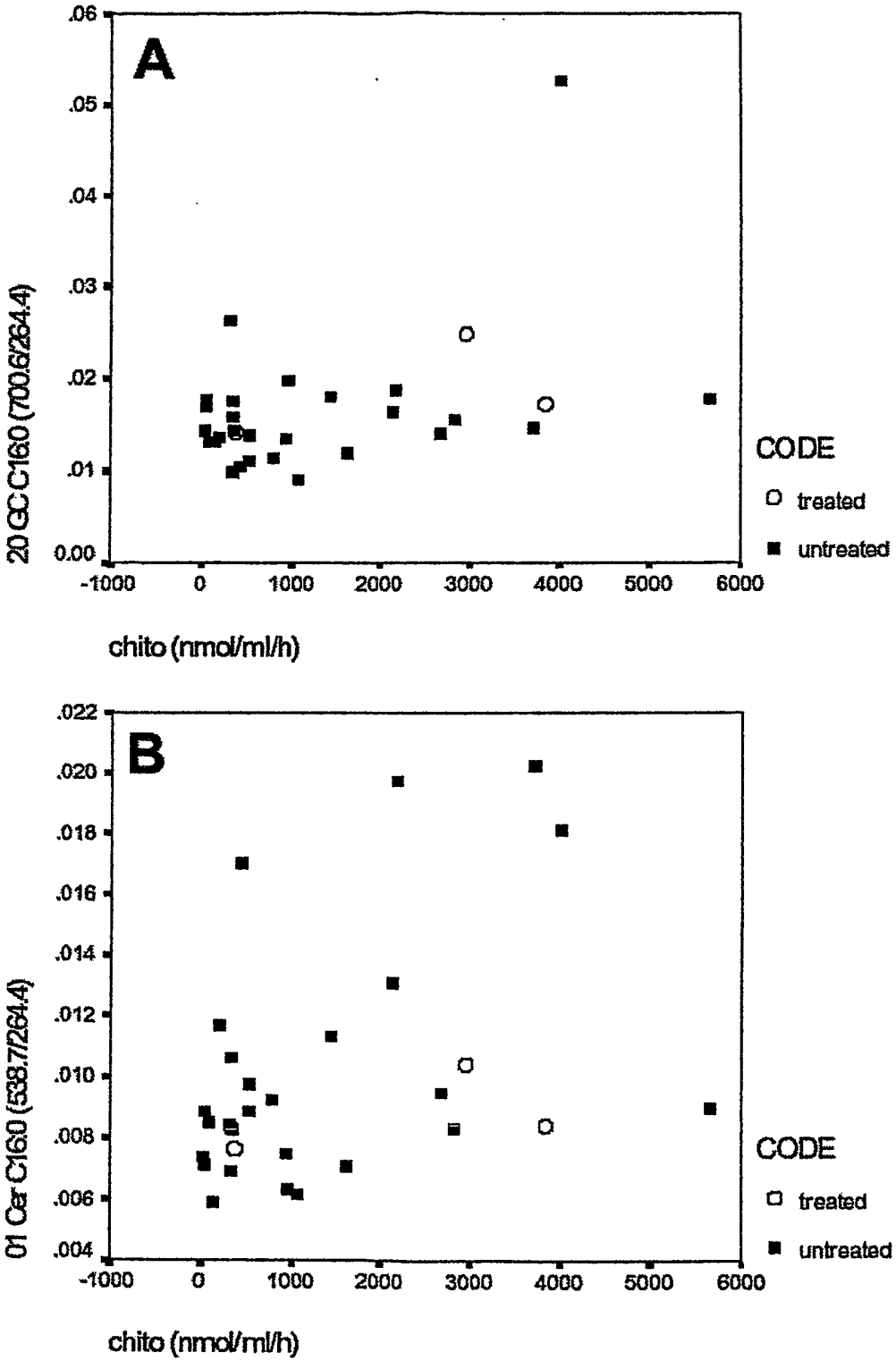
A
.06
.05
.04
.03
.02
.01
0.00
20 GCC160 (700.6/264.4)
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated
B
.022
.020
.018
.016
.014
.012
.010
.008
.006
.004
01 Cer C16:0 (538.7/264.4)
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated

FIGURE 7

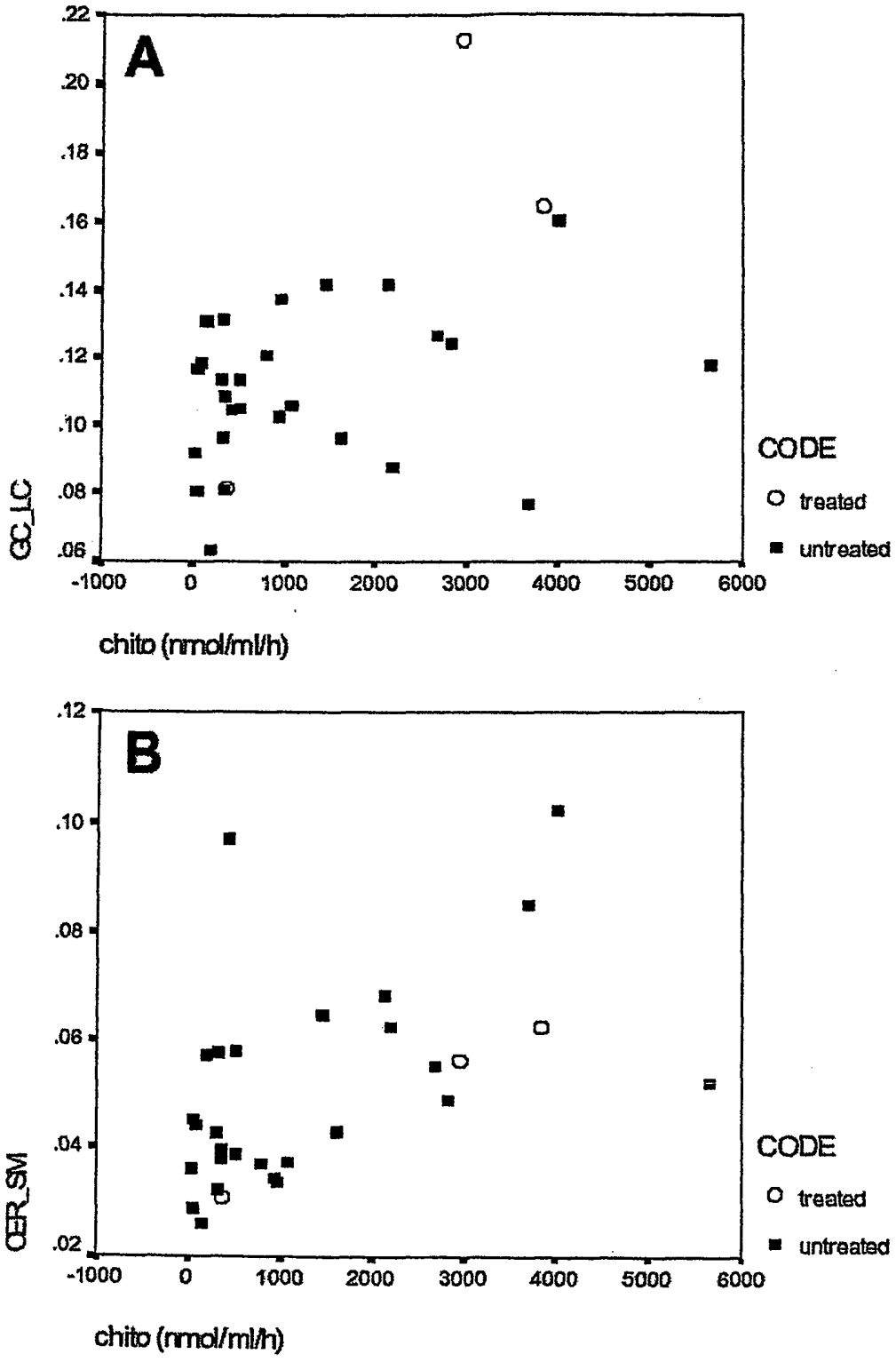
A
.22
.20
.18
.16
.14
.12
.10
.08
.06
GC_LC
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated
B
.12
.10
.08
.06
.04
.02
CER_SM
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated

FIGURE 8

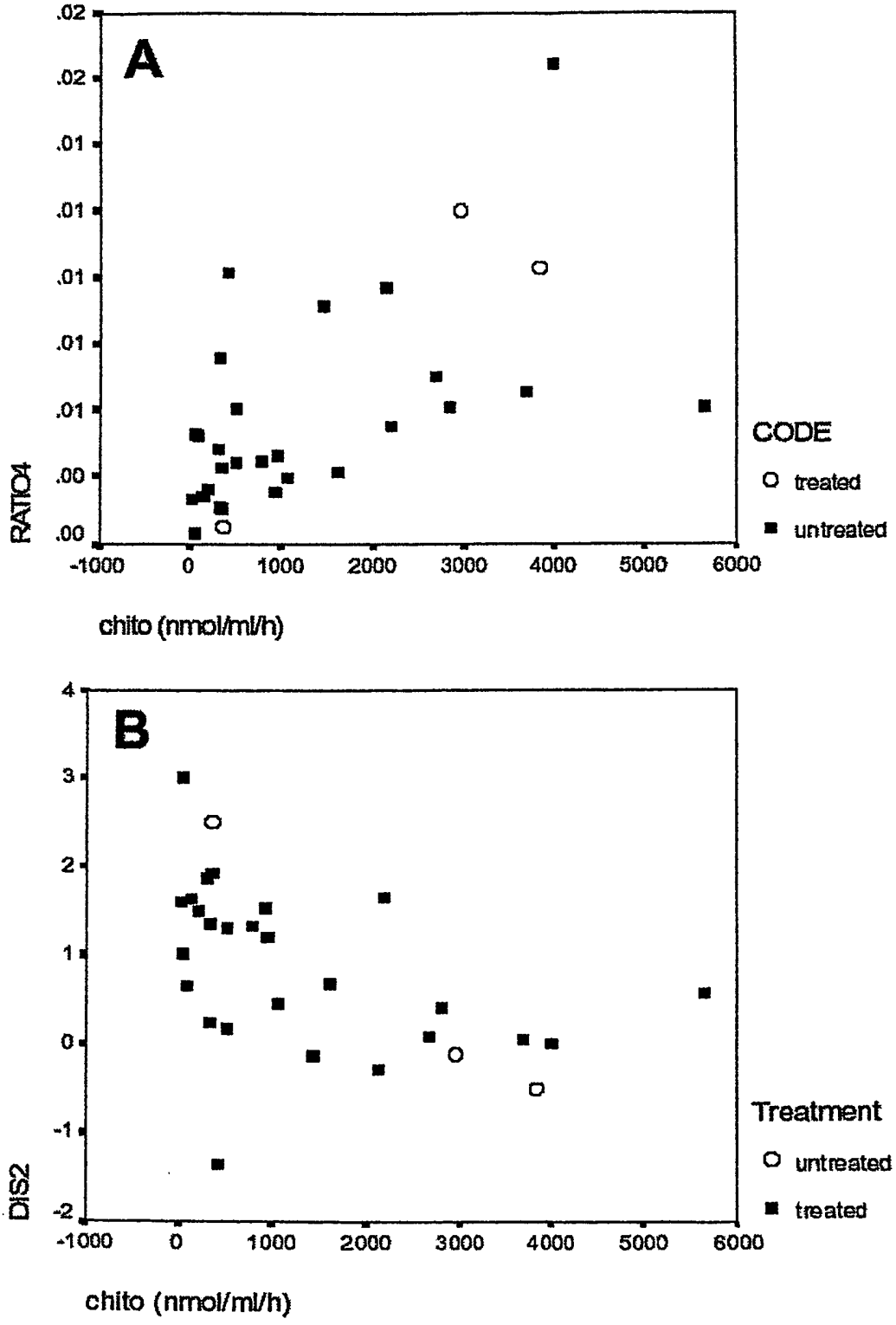
A
.02
.02
.01
.01
.01
.01
.01
.00
.00
RATIO4
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
CODE
treated
untreated
B
4
3
2
1
0
-1
-2
DIS2
-1000
0
1000
2000
3000
4000
5000
6000
chito (nmol/ml/h)
Treatment
untreated
treated

FIGURE 9

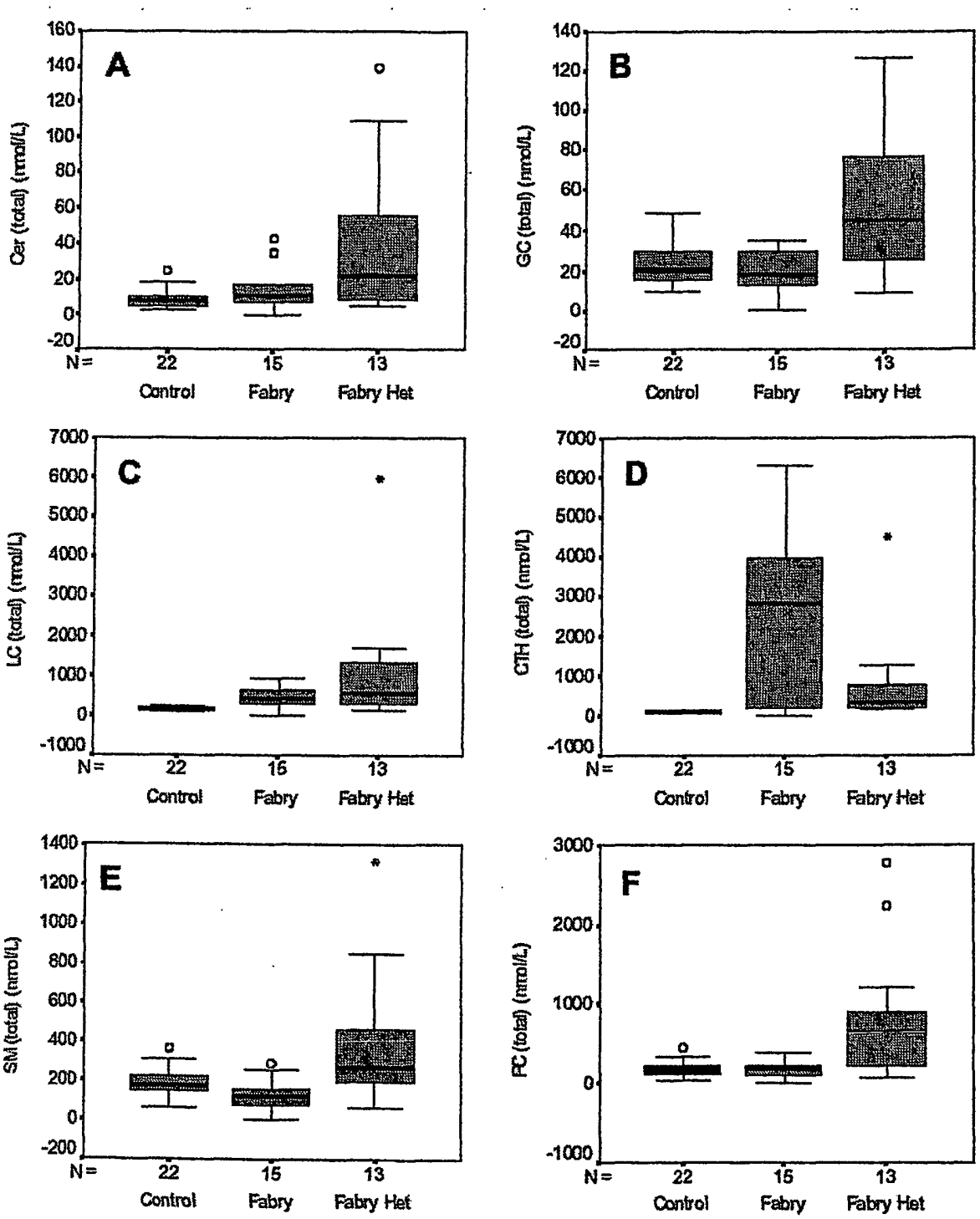
A
Cer (total) (nmol/L)
160
140
120
100
80
60
40
20
0
-20
N=
22
15
13
Control
Fabry
Fabry Het
B
GC (total) (nmol/L)
140
120
100
80
60
40
20
0
-20
N=
22
15
13
Control
Fabry
Fabry Het
C
LC (total) (nmol/L)
7000
6000
5000
4000
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het
D
CTH (total) (nmol/L)
7000
6000
5000
4000
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het
E
SM (total) (nmol/L)
1400
1200
1000
800
600
400
200
0
-200
N=
22
15
13
Control
Fabry
Fabry Het
F
FC (total) (nmol/L)
3000
2000
1000
0
-1000
N=
22
15
13
Control
Fabry
Fabry Het

FIGURE 10

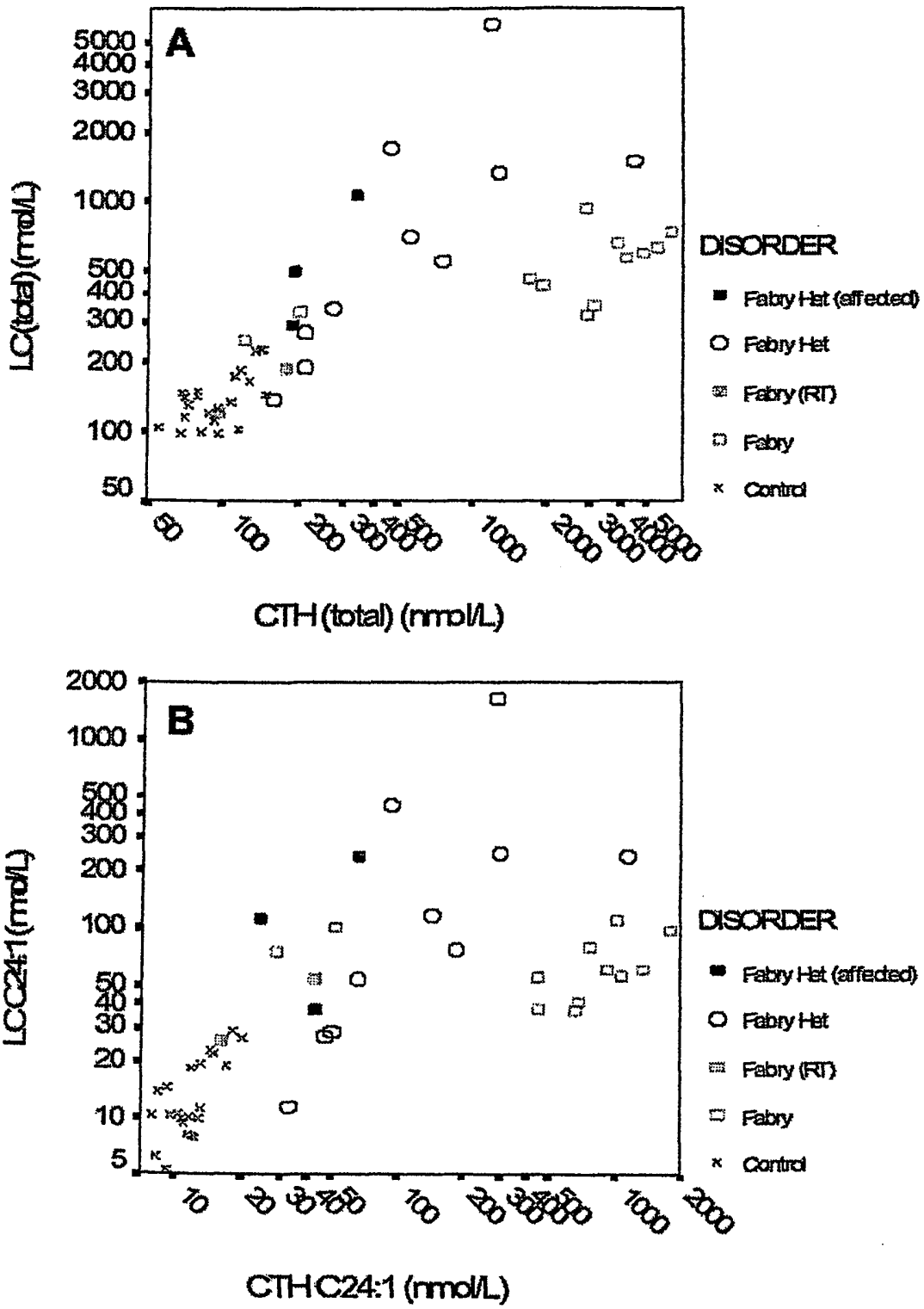
A
LC(total)(nmol/L)
5000
4000
3000
2000
1000
500
400
300
200
100
50
50
100
200
300
400
500
1000
2000
3000
4000
5000
CTH (total) (nmol/L)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control
B
LCC24:1 (nmol/L)
2000
1000
500
400
300
200
100
50
40
30
20
10
5
10
20
30
40
50
100
200
300
400
500
1000
2000
CTH C24:1 (nmol/L)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control

FIGURE 11

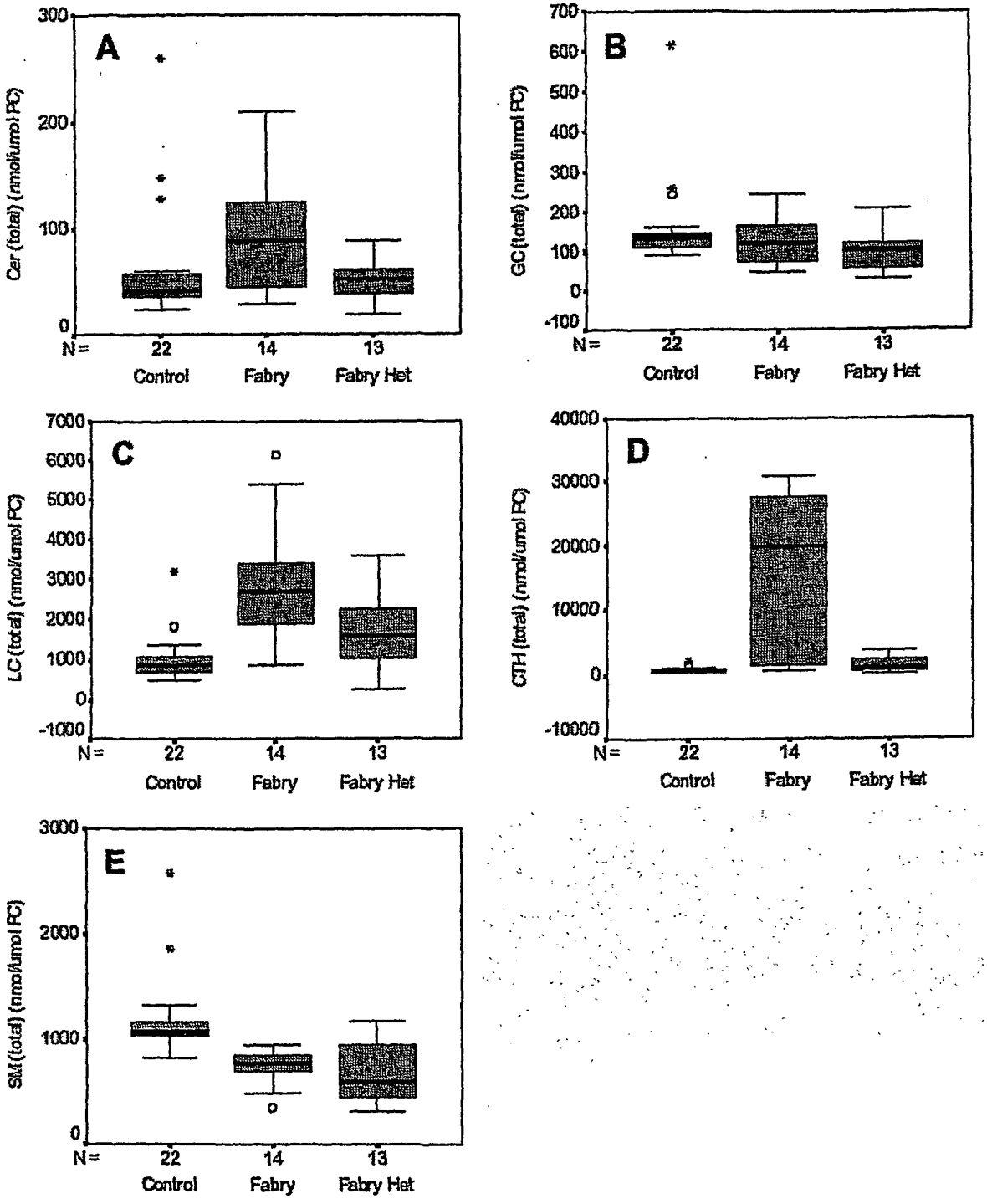
A
Cer (total) (nmol/umol PC)
300
200
100
0
N=
22
14
13
Control
Fabry
Fabry Het
B
GC (total) (nmol/umol PC)
700
600
500
400
300
200
100
0
-100
C
LC (total) (nmol/umol PC)
7000
6000
5000
4000
3000
2000
1000
0
-1000
D
CTH (total) (nmol/umol PC)
40000
30000
20000
10000
0
-10000
E
SM (total) (nmol/umol PC)
3000
2000
1000
0

FIGURE 12

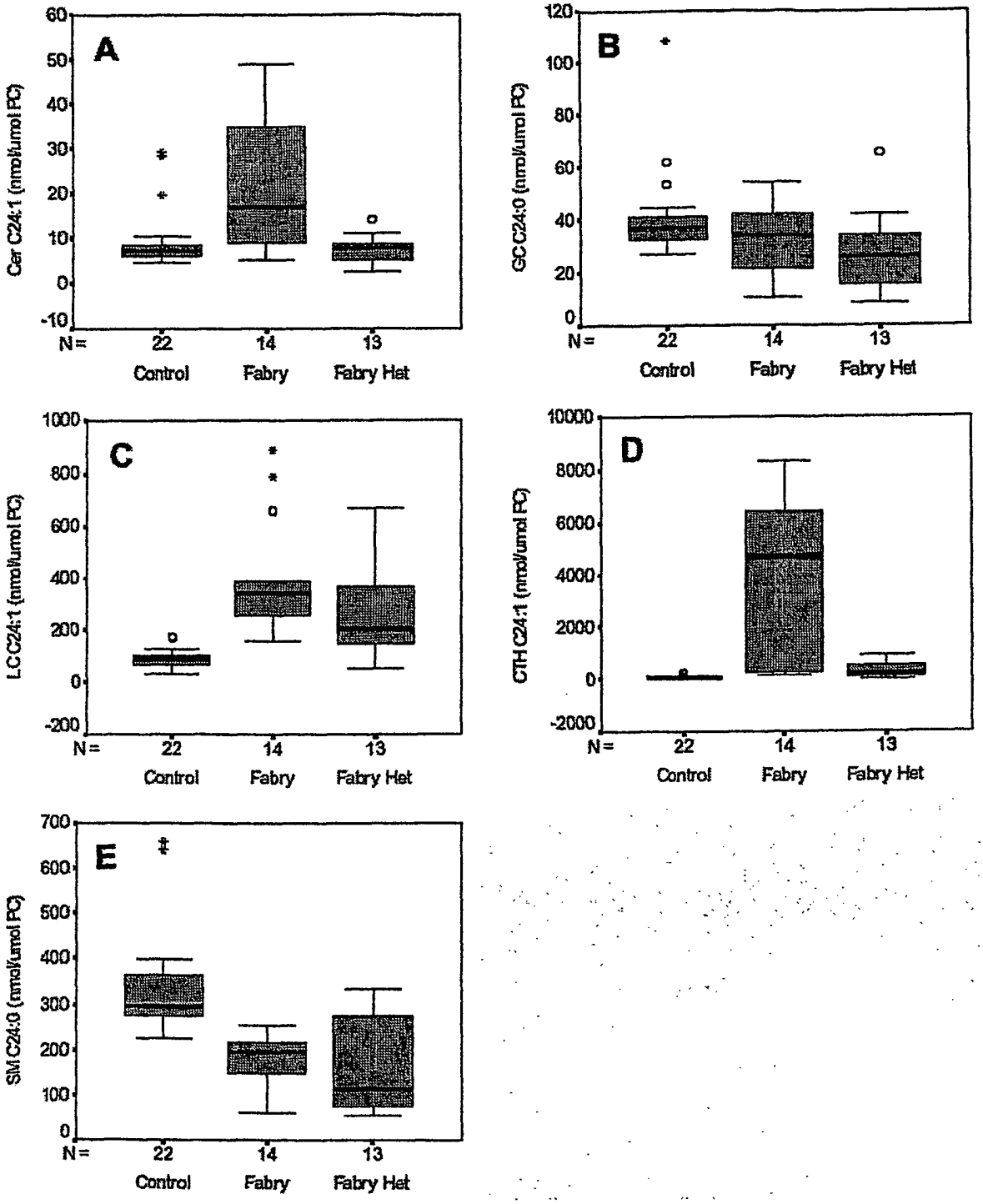
A
Cer C24:1 (nmol/umol PC)
60
50
40
30
20
10
0
-10
N=
22
14
13
Control
Fabry
Fabry Het
B
GCC24:0 (nmol/umol PC)
120
100
80
60
40
20
0
C
LCC24:1 (nmol/umol PC)
1000
800
600
400
200
0
-200
D
CTH C24:1 (nmol/umol PC)
10000
8000
6000
4000
2000
0
-2000
E
SM C24:0 (nmol/umol PC)
700
600
500
400
300
200
100
0

FIGURE 13

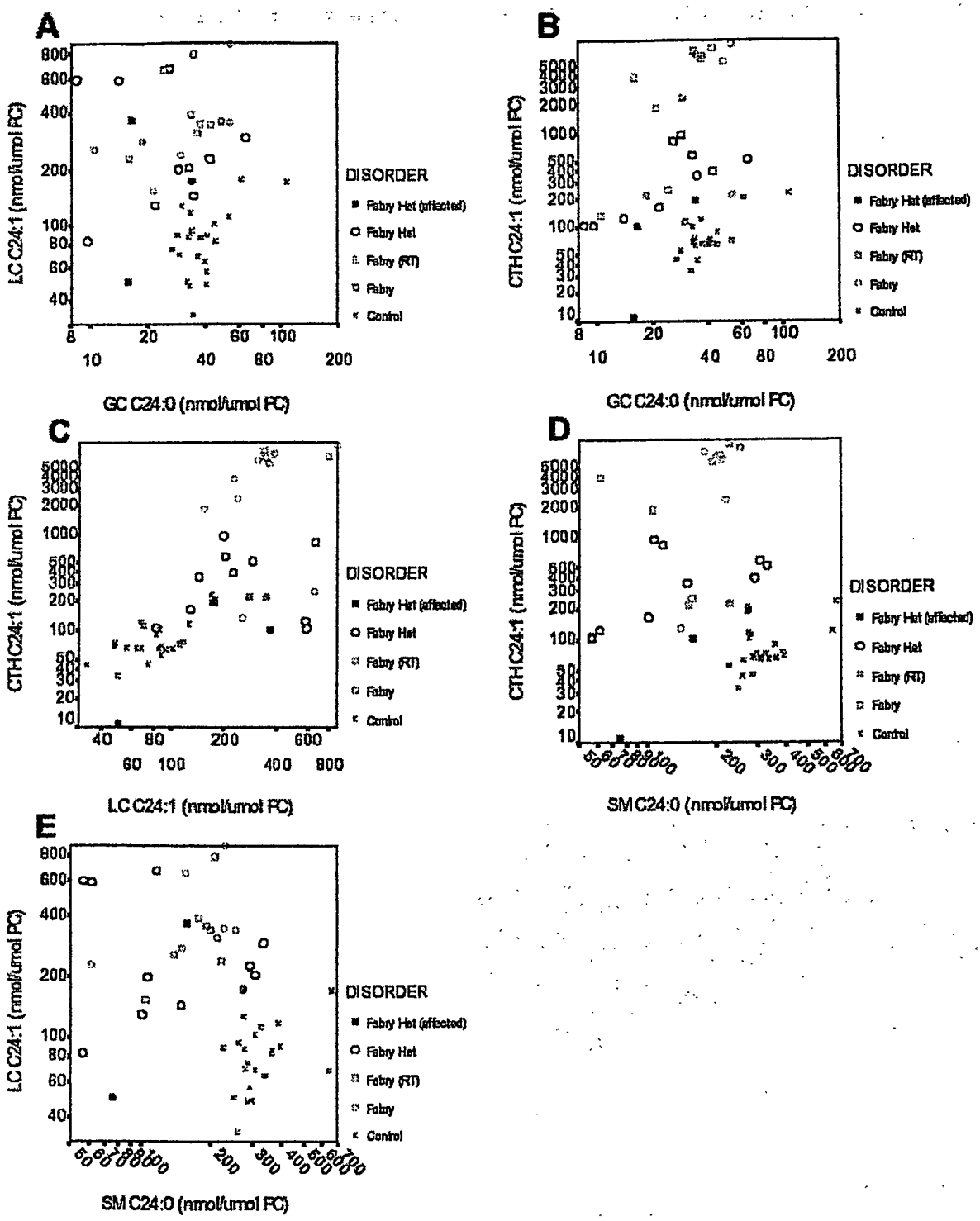
A
LC C24:1 (nmol/umol PC)
GC C24:0 (nmol/umol PC)
B
CTH C24:1 (nmol/umol PC)
GC C24:0 (nmol/umol PC)
C
CTH C24:1 (nmol/umol PC)
LC C24:1 (nmol/umol PC)
D
CTH C24:1 (nmol/umol PC)
SM C24:0 (nmol/umol PC)
E
LC C24:1 (nmol/umol PC)
SM C24:0 (nmol/umol PC)
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control

FIGURE 14

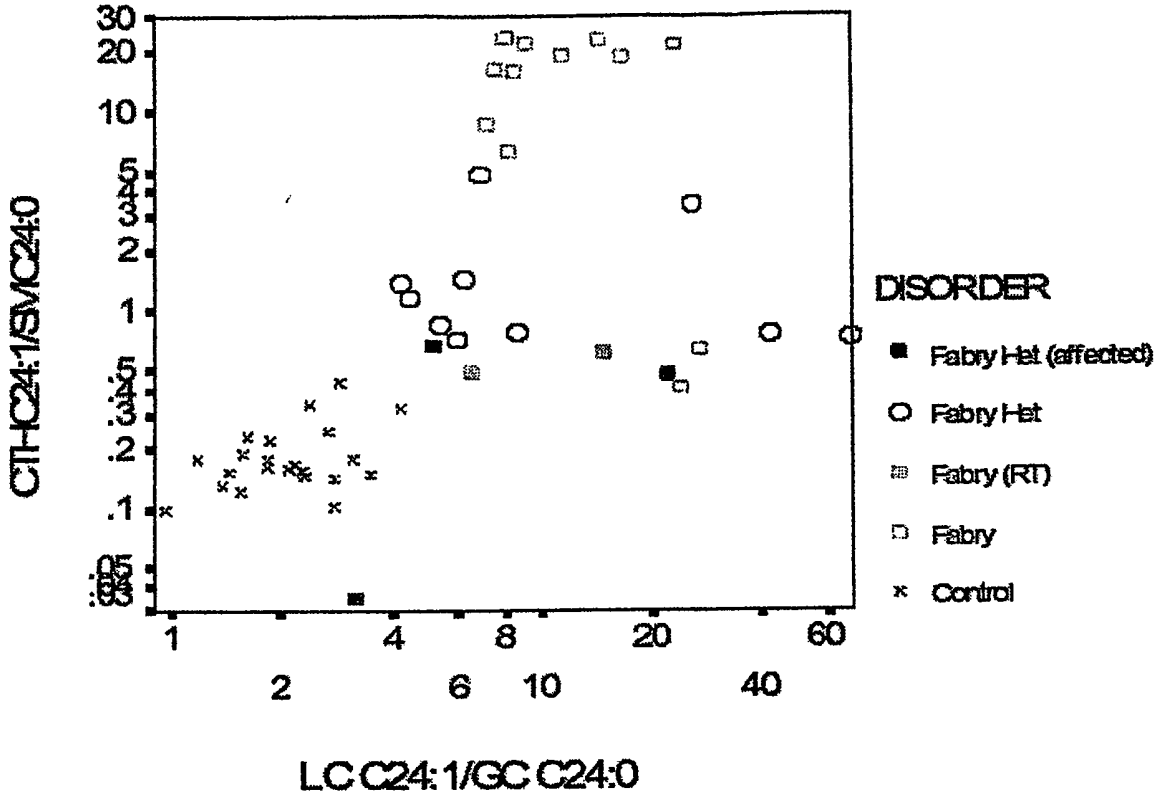
CTHC24:1/SMC24:0
30
20
10
5
2
1
.1
1
2
4
6
8
10
20
40
60
LC C24:1/GC C24:0
DISORDER
Fabry Het (affected)
Fabry Het
Fabry (RT)
Fabry
Control

FIGURE 15

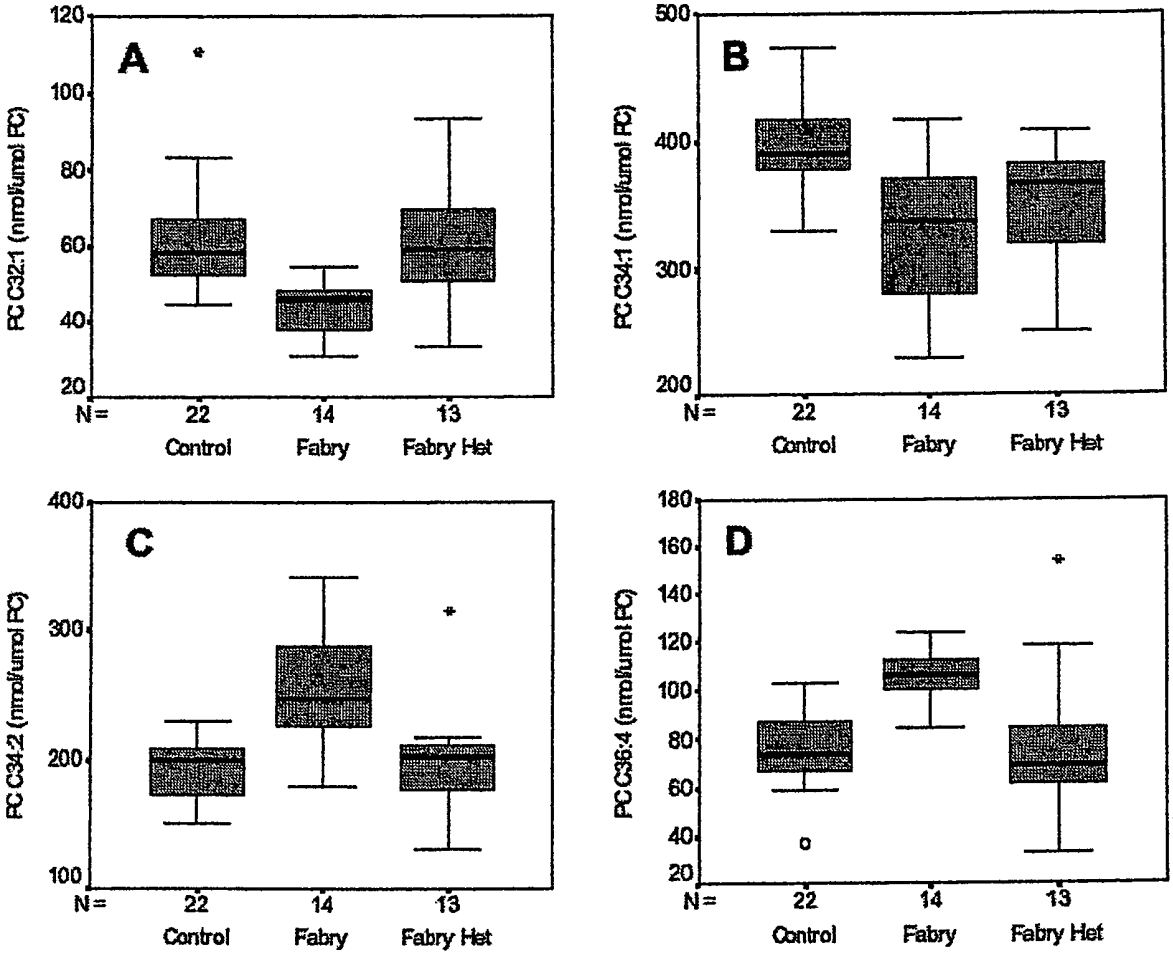
A
PC C32:1 (nmol/umol PC)
120
100
80
60
40
20
N=
22
14
13
Control
Fabry
Fabry Het
B
PC C34:1 (nmol/umol PC)
500
400
300
200
C
PC C34:2 (nmol/umol PC)
400
300
200
100
D
PC C36:4 (nmol/umol PC)
180
160
140
120
100
80
60
40
20

FIGURE 16

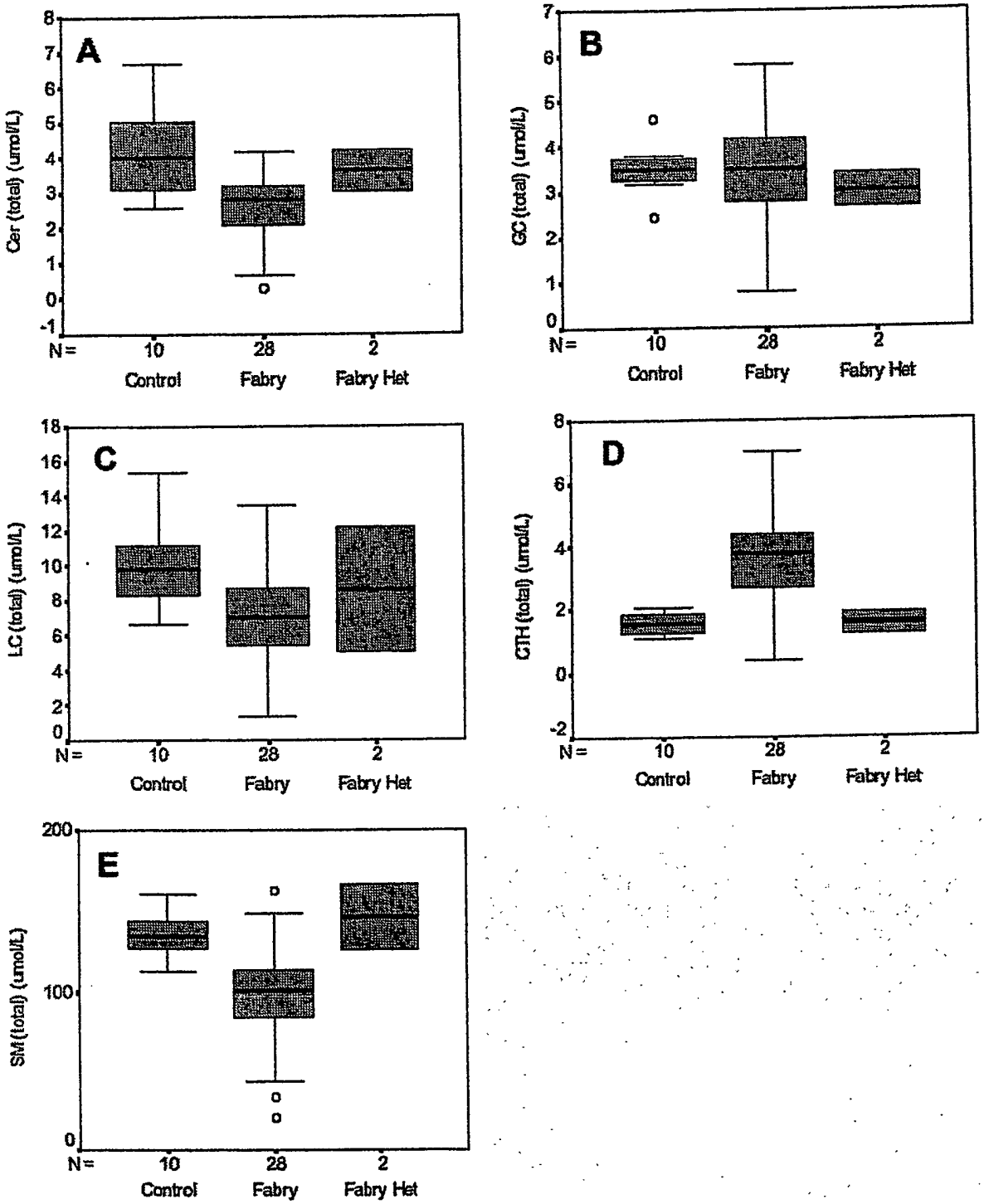
A
Cer (total) (umol/L)
N=
10
28
2
Control
Fabry
Fabry Het
B
GC (total) (umol/L)
C
LC (total) (umol/L)
D
GTH (total) (umol/L)
E
SM (total) (umol/L)

FIGURE 17

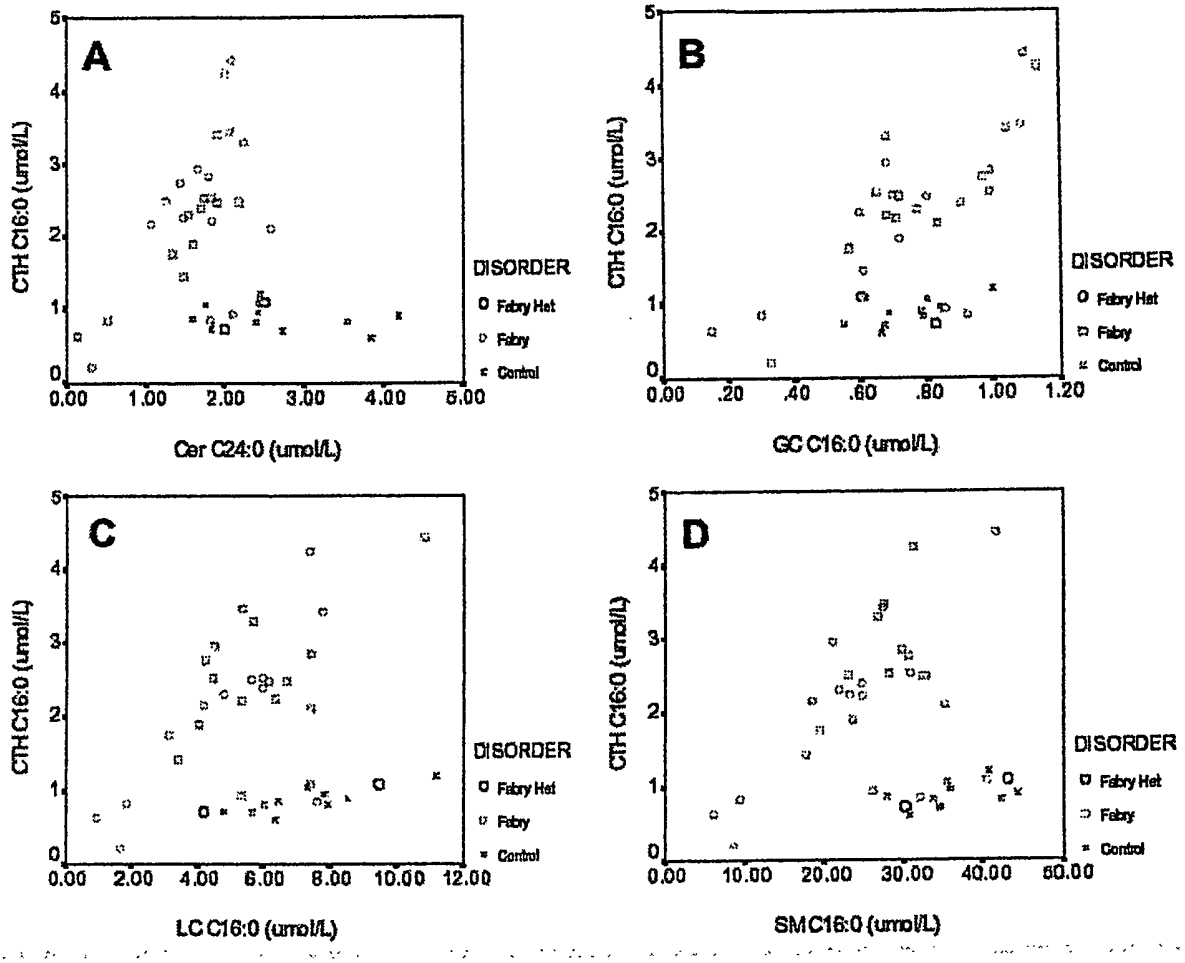
A
CTH C16:0 (umol/L)
Cer C24:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
B
CTH C16:0 (umol/L)
GC C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
C
CTH C16:0 (umol/L)
LC C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
D
CTH C16:0 (umol/L)
SM C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control

FIGURE 18

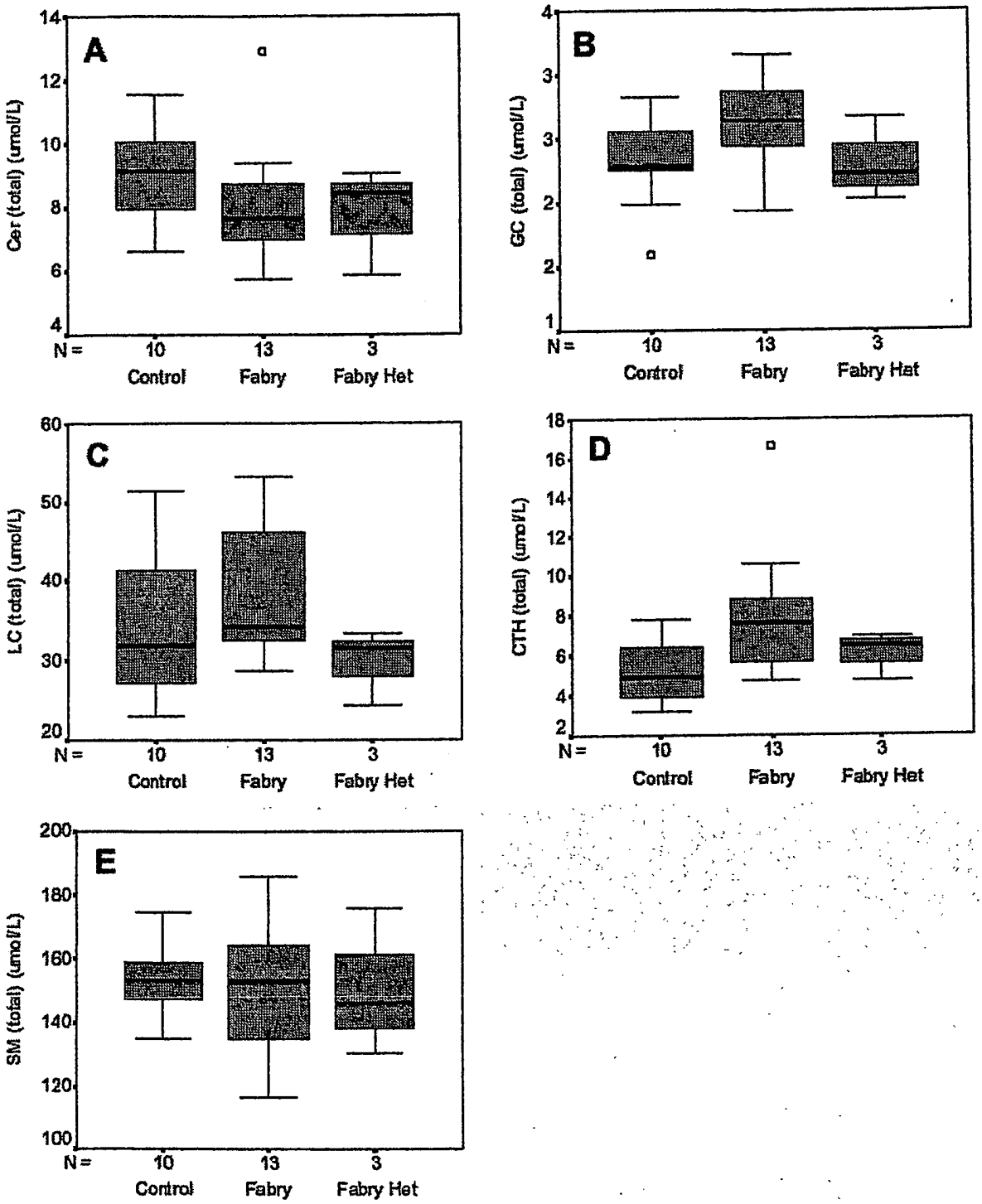
A
Cer (total) (umol/L)
14
12
10
8
6
4
N =
10
13
3
Control
Fabry
Fabry Het
B
GC (total) (umol/L)
4
3
3
2
2
1
N =
10
13
3
Control
Fabry
Fabry Hat
C
LC (total) (umol/L)
60
50
40
30
20
N =
10
13
3
Control
Fabry
Fabry Het
D
CTH (total) (umol/L)
18
16
14
12
10
8
6
4
2
N =
10
13
3
Control
Fabry
Fabry Het
E
SM (total) (umol/L)
200
180
160
140
120
100
N =
10
13
3
Control
Fabry
Fabry Het

FIGURE 19

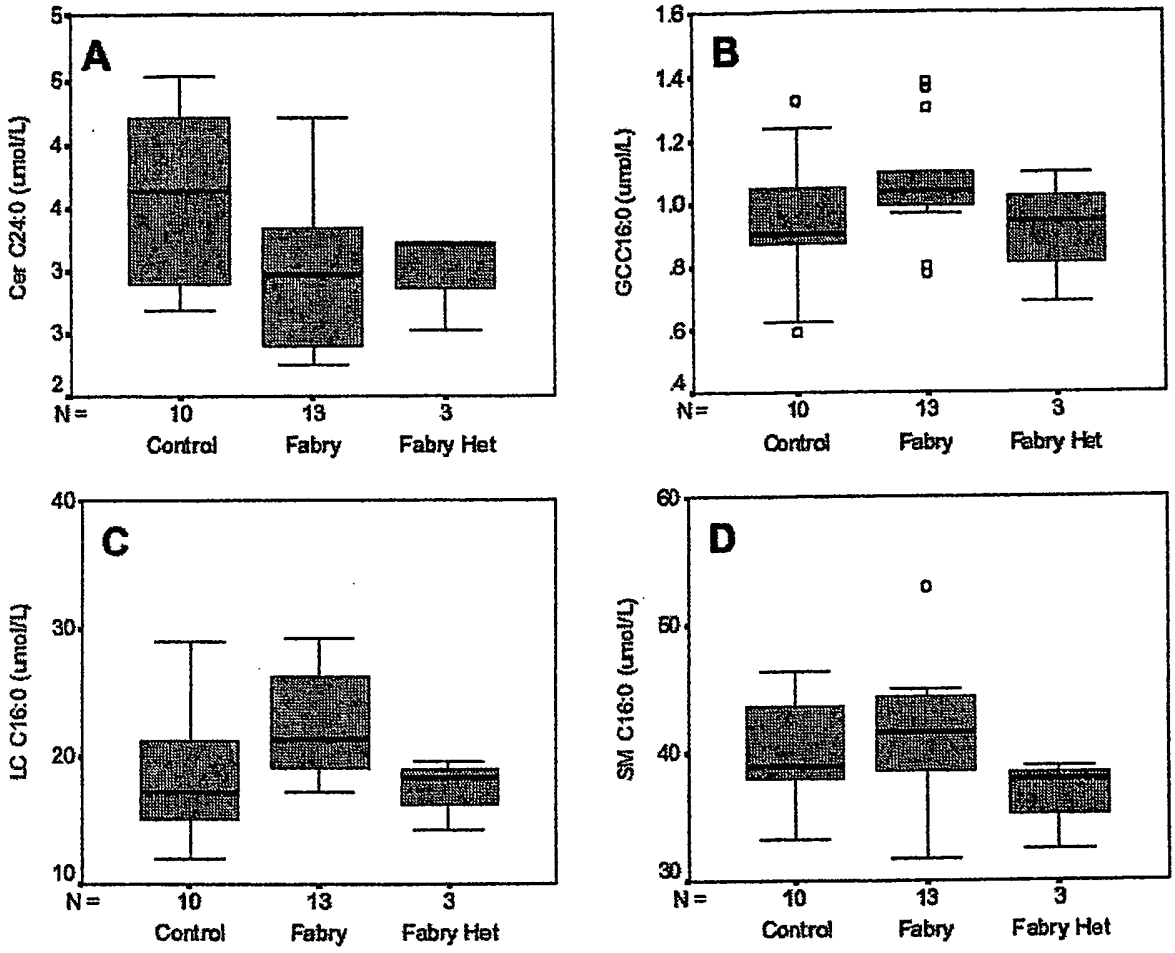
A
Cer C24:0 (umol/L)
5
6
4
4
3
3
2
N=
10
13
3
Control
Fabry
Fabry Het
B
GCC16:0 (umol/L)
1.6
1.4
1.2
1.0
.8
.6
.4
C
LC C16:0 (umol/L)
40
30
20
10
D
SM C16:0 (umol/L)
60
50
40
30

FIGURE 20

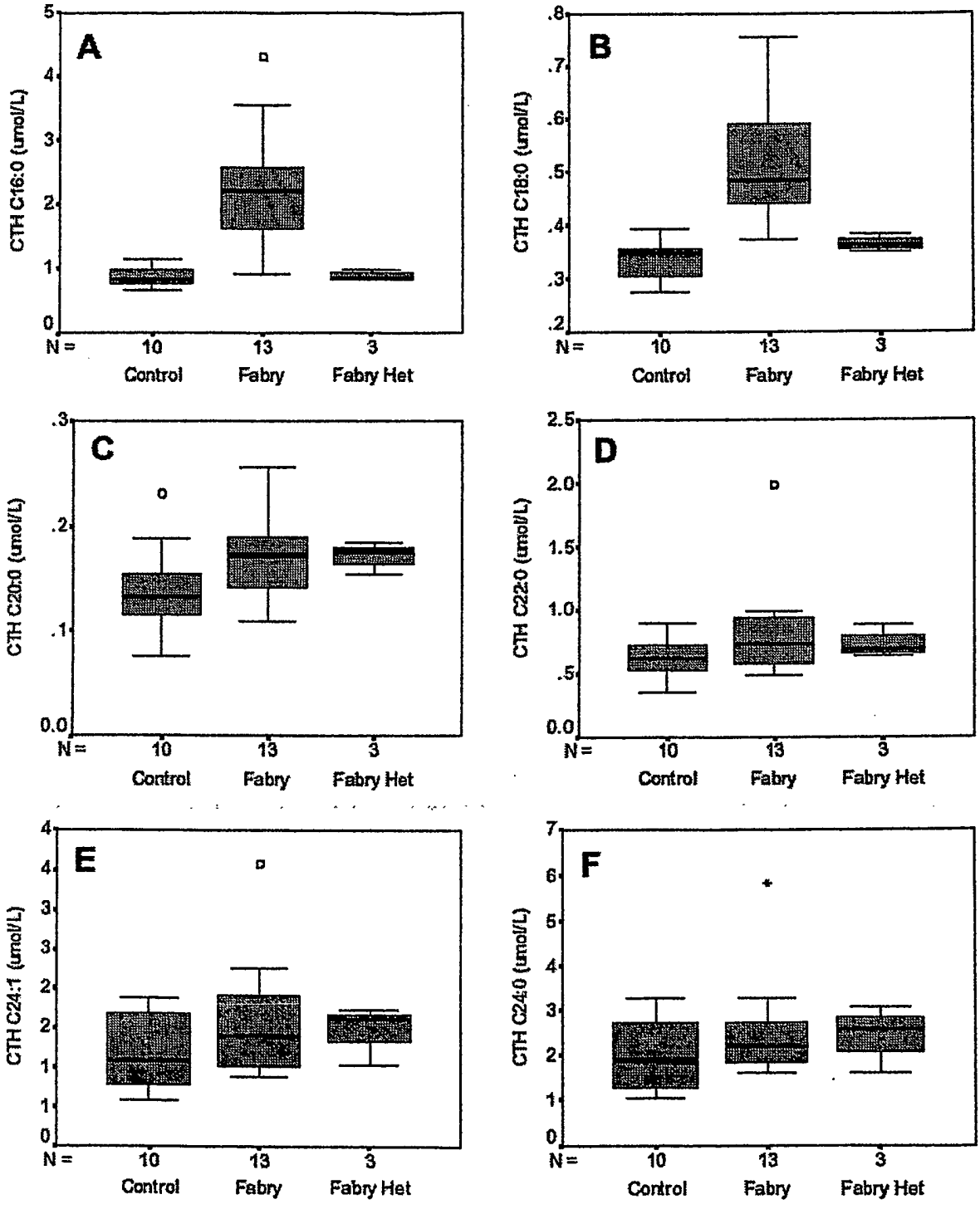
A
CTH C16:0 (umol/L)
N = 10 13 3
Control Fabry Fabry Het
B
CTH C18:0 (umol/L)
N = 10 13 3
Control Fabry Fabry Het
C
CTH C20:0 (umol/L)
N = 10 13 3
Control Fabry Fabry Het
D
CTH C22:0 (umol/L)
N = 10 13 3
Control Fabry Fabry Het
E
CTH C24:1 (umol/L)
N = 10 13 3
Control Fabry Fabry Het
F
CTH C24:0 (umol/L)
N = 10 13 3
Control Fabry Fabry Het

FIGURE 21

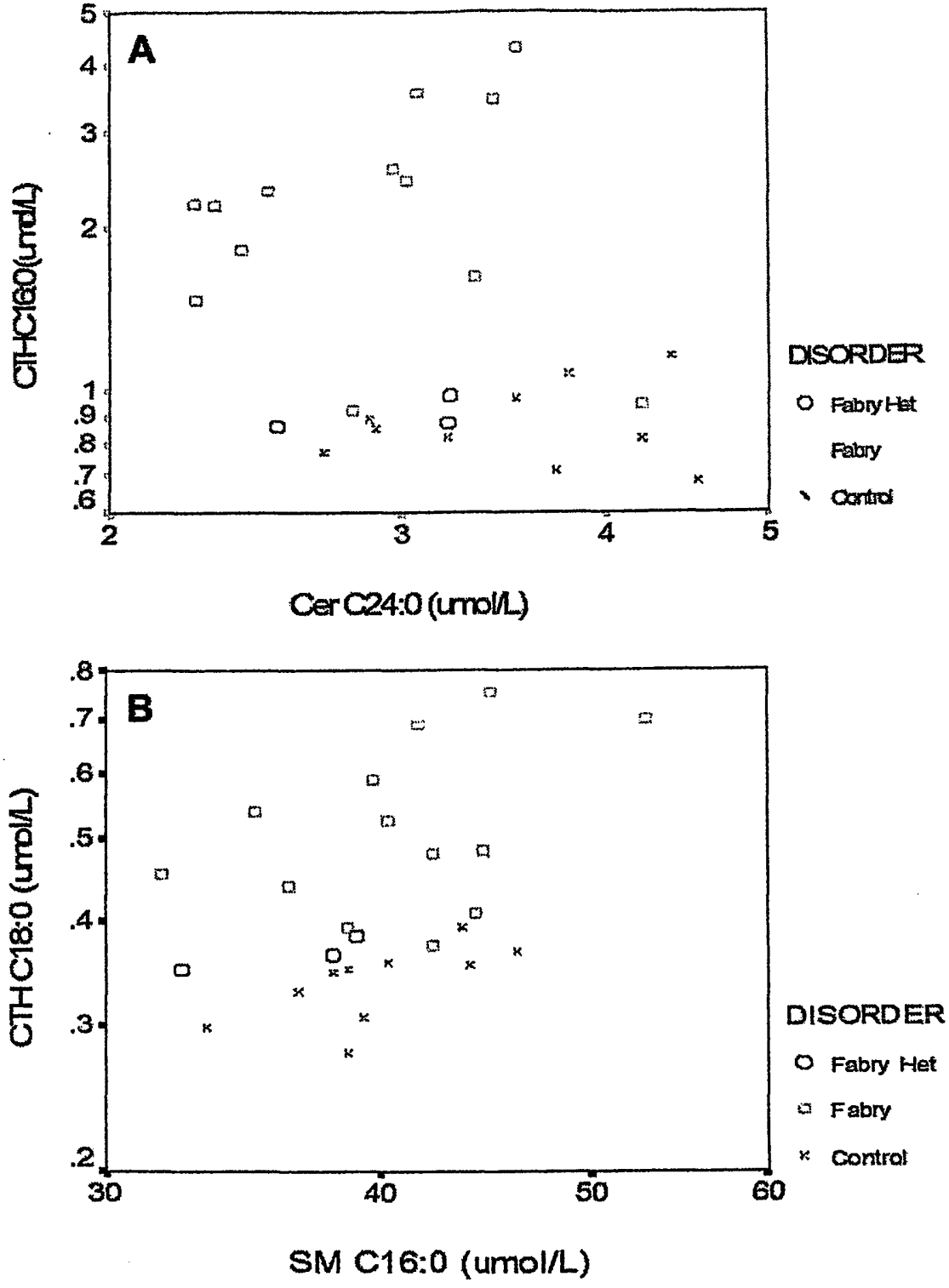
A
CTH C16:0 (umol/L)
Cer C24:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control
B
CTH C18:0 (umol/L)
SM C16:0 (umol/L)
DISORDER
Fabry Het
Fabry
Control

FIGURE 22

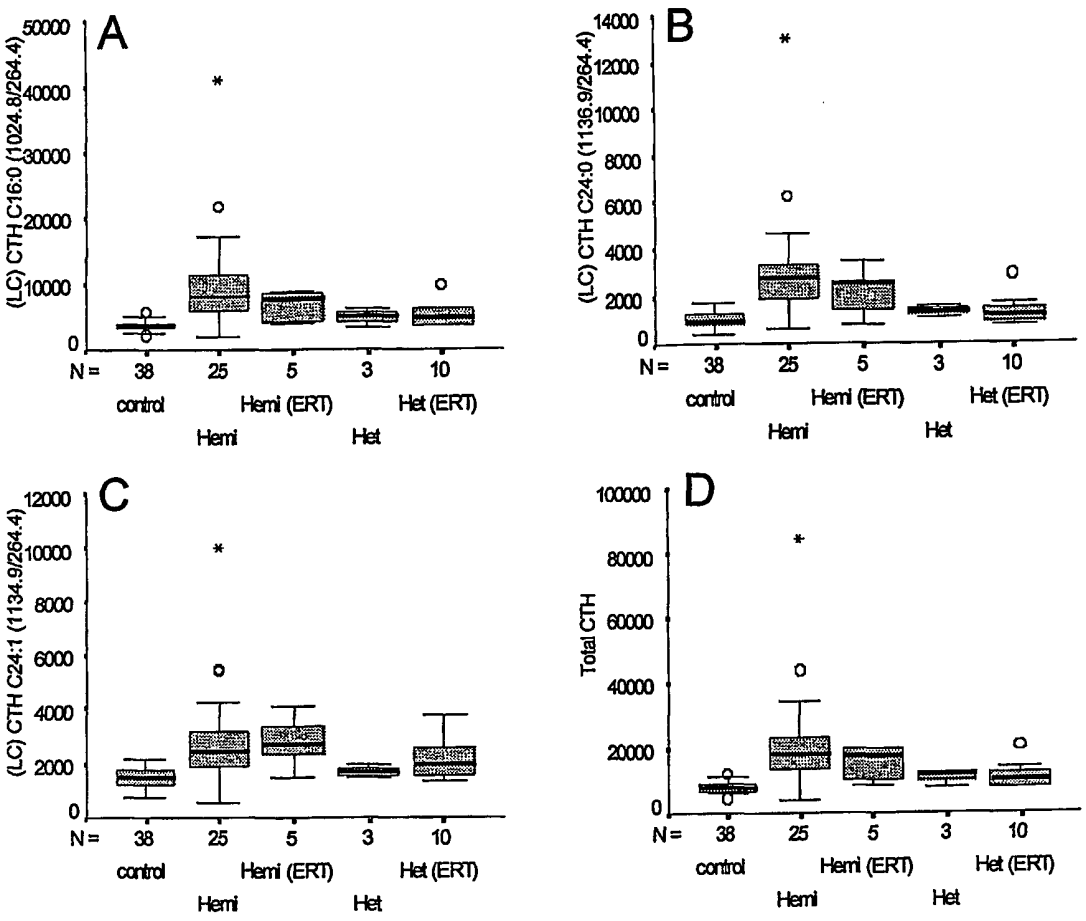
A
(LC) CTH C16:0 (1024.8/264.4)
50000
40000
30000
20000
10000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
B
(LC) CTH C24:0 (1136.9/264.4)
14000
12000
10000
8000
6000
4000
2000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
C
(LC) CTH C24:1 (1134.9/264.4)
12000
10000
8000
6000
4000
2000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
D
Total CTH
100000
80000
60000
40000
20000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het

Figure 23

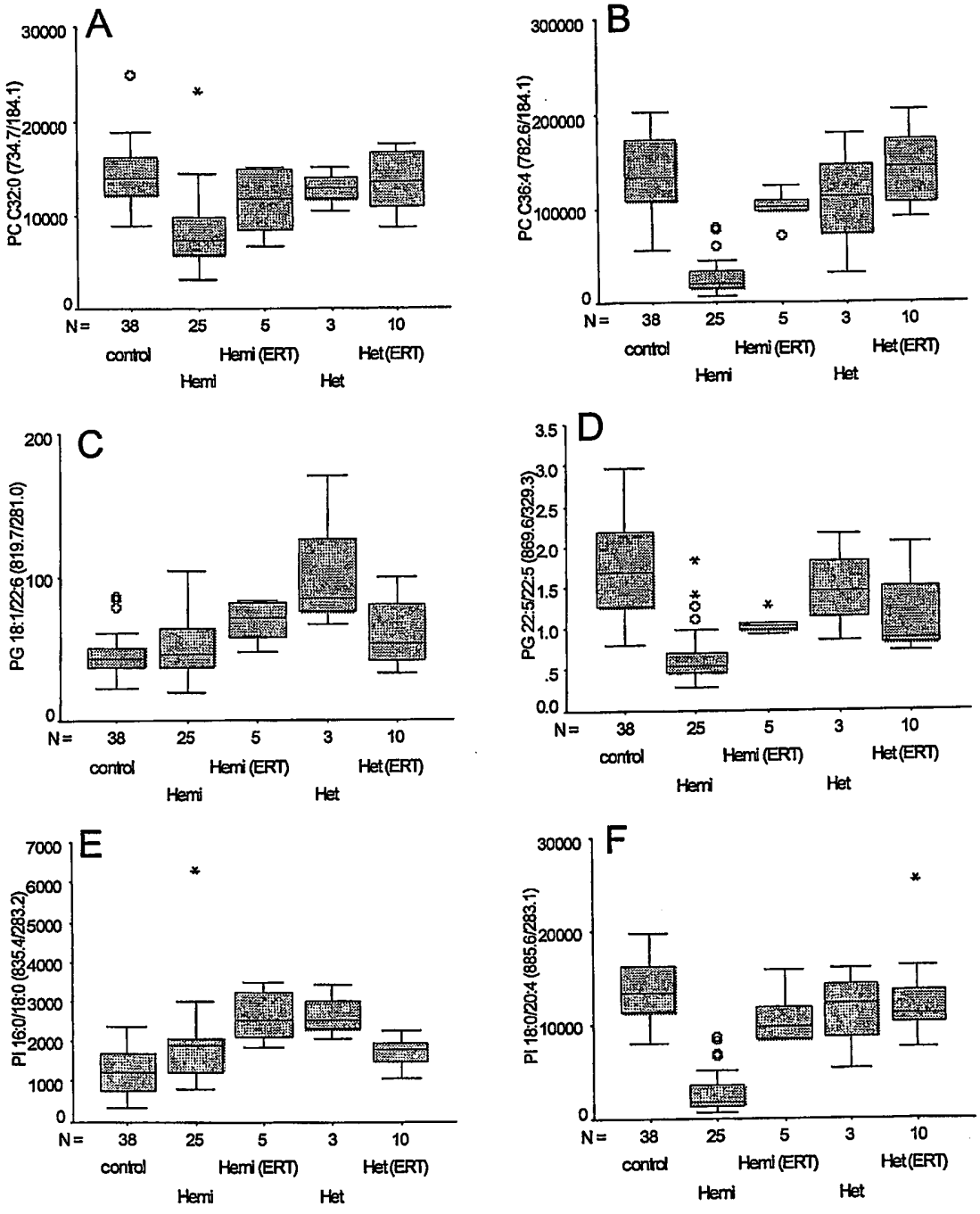
A
PC C32:0 (734.7/184.1)
30000
20000
10000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
B
PC C36:4 (782.6/184.1)
300000
200000
100000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
C
PG 18:1/22:6 (819.7/281.0)
200
100
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
D
PG 22:5/22:5 (869.6/329.3)
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
E
PI 16:0/18:0 (835.4/283.2)
7000
6000
5000
4000
3000
2000
1000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het
F
PI 18:0/20:4 (885.6/283.1)
30000
20000
10000
0
N= 38 25 5 3 10
control Hemi (ERT) Het (ERT)
Hemi Het

Figure 24

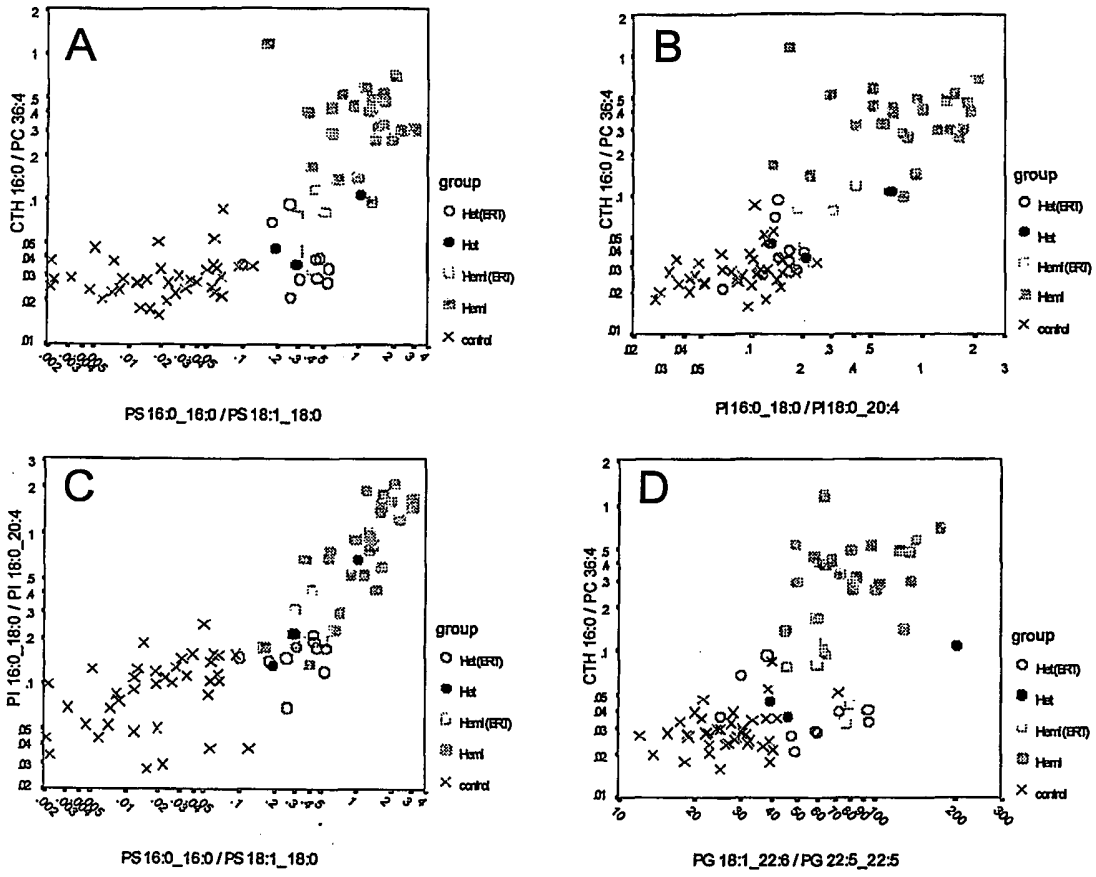
A
CTH 16:0 / PC 36:4
PS 16:0_16:0 / PS 18:1_18:0
group
Het(ERI)
Het
Hom(ERI)
Hom
control
B
CTH 16:0 / PC 36:4
PI 16:0_18:0 / PI 18:0_20:4
group
Het(ERI)
Het
Hom(ERI)
Hom
control
C
PI 16:0_18:0 / PI 18:0_20:4
PS 16:0_16:0 / PS 18:1_18:0
group
Het(ERI)
Het
Hom(ERI)
Hom
control
D
CTH 16:0 / PC 36:4
PG 18:1_22:6 / PG 22:5_22:5
group
Het(ERI)
Het
Hom(ERI)
Hom
control

Figure 25

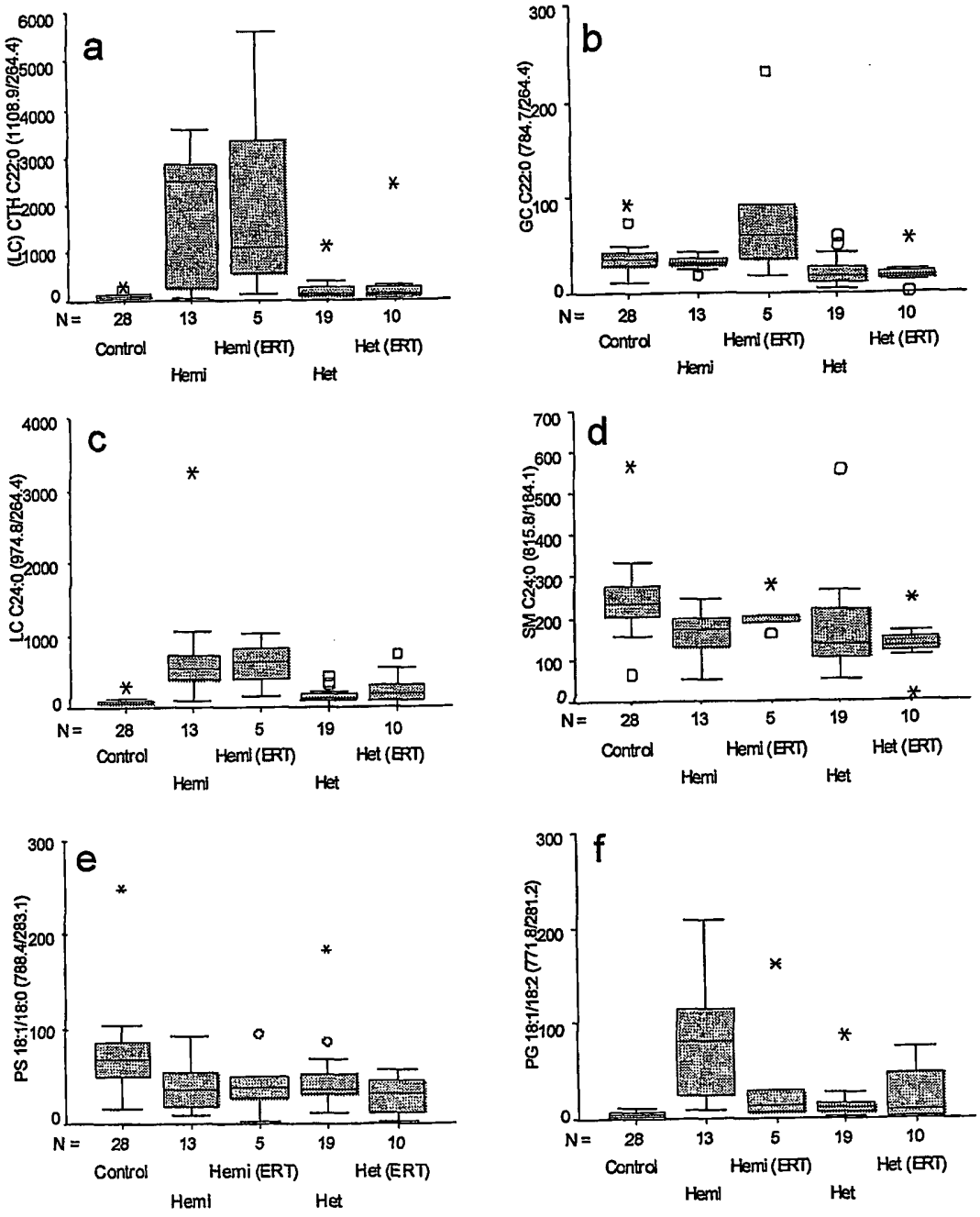
a
(LC) CTH C22:0 (1108.9/264.4)
6000
5000
4000
3000
2000
1000
0
N= 28 13 5 19 10
Control
Hemi
Hemi (ERT)
Het
Het (ERT)
b
GC C22:0 (784.7/264.4)
300
200
100
0
c
LC C24:0 (974.8/264.4)
4000
3000
2000
1000
d
SM C24:0 (815.8/184.1)
700
600
500
400
300
200
100
e
PS 18:1/18:0 (788.4/283.1)
f
PG 18:1/18:2 (771.8/281.2)

Figure 26

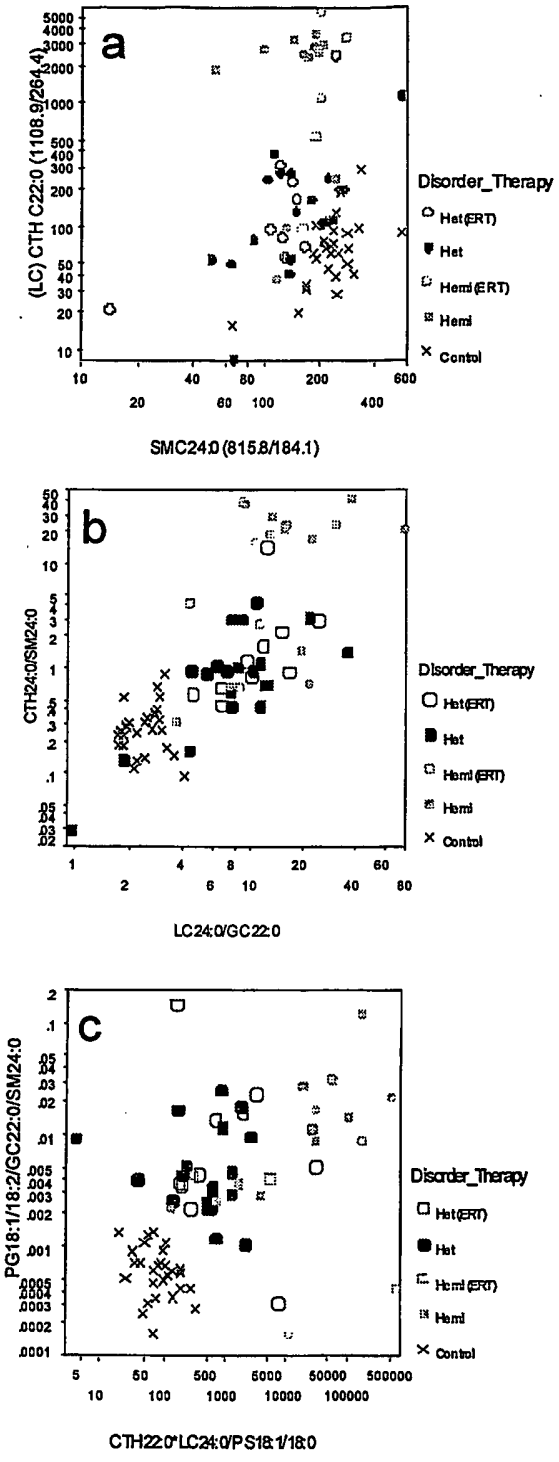
a
(LC) CTH C22:0 (1108.9/264.4)
SMC24:0 (815.8/184.1)
Disorder_Therapy
Het(ERT)
Het
Heml(ERT)
Heml
Control
b
CTH24:0/SM24:0
LC24:0/GC22:0
Disorder_Therapy
Het(ERT)
Het
Heml(ERT)
Heml
Control
c
PG18:1/18:2/GC22:0/SM24:0
CTH22:0*LC24:0/PS18:1/18:0
Disorder_Therapy
Het(ERT)
Het
Heml(ERT)
Heml
Control

Figure 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000461

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: G01N 33/50 G01N 33/92

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, WPIDS, BIOSIS: lipid, glycolipid, lysosomal, disorder, disease, Gaucher, Fabry, ratio, detect, diagnose

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Whitfield PD *et al* (2002) *Mol. Gen & Metabolism* 75: 46-55 "Correlation among Genotype, Phenotype and Biochemical Markers in Gaucher Disease: Implications for the Prediction of Disease Severity" Abstract, Fig 1 and Fig 3 Also cited by Applicants	1, 2, 4 – 16 & 23 -27
X	Cable WJL *et al* (1982) *Neurology* (Ny) **32**: 1139-1145 "Fabry disease: Detection of heterozygotes by examination of glycolipids in urinary sediment" Abstract, Figure 2 and Table page 1143	1, 2, 4-8, 11-14, & 16 -21

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 May 2005	19 MAY 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **Philippa Wyrdeman** Telephone No : (02) 6283 2554

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000461

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Fujiwaki T *et al* (2002) *Brain & Development* **24**:170-173 "Application of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry for analysis of sphingolipids in cultured skin fibroblasts from sphingolipidosis patients"	
X	Abstract, Figure 2	1-9, 11, 13, 14-16,& 23-27
	Fujiwaki T *et al* (2002) *J. Chromatography B* **776**: 115-123 "Application of delayed extraction-matrix-assisted laser desorption ionization time-of-flight mass spectrometry for analysis of sphingolipids in pericardial fluid, peritoneal fluid and serum from Gaucher disease patients"	
X	Abstract, Figure 5	1, 2, 4-9, 11-16 & 23-27
	Oshima M *et al* (1990) *Bioch et Biophys Acta* **1043**: 157-160 "Urinary neutral glycosphingolipid analysis of patients with Fabry's disease; rapid isocratic elution from high-performance liquid chromatrography as per-*o*-benzoyl derivatives"	
X	Abstract Table I	1, 2, 4-8, 11, 13, 14, 17-21